

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Diane M. Ruezinsky

Serial No.: 10/810,788

Filed: March 26, 2004

For: NOVEL PLANT PROMOTERS FOR USE
IN EARLY SEED DEVELOPMENT

Group Art Unit: 1638

Examiner: Page, Brent T.

Atty. Dkt. No.: MONS:060US

BRIEF ON APPEAL

TABLE OF CONTENTS

I.	REAL PARTY IN INTEREST	2
II.	RELATED APPEALS AND INTERFERENCES	2
III.	STATUS OF THE CLAIMS	2
IV.	STATUS OF AMENDMENTS	2
V.	SUMMARY OF CLAIMED SUBJECT MATTER	3
VI.	GROUND OF REJECTION TO BE REVIEWED ON APPEAL	3
VII.	ARGUMENT	3
A.	Substantially Similar Issues Were Decided by the Board in Appeal No. 2003-0936	3
B.	The Claims Comply with the Written Description Requirement Under 35 U.S.C. §112, First Paragraph	9
1.	Written Description Must Be Analyzed with Respect to the Claimed Invention.....	9
2.	Modification of Promoter Sequences is Routine in the Art.....	12
C.	The Claims Are Enabled Under 35 U.S.C. §112, First Paragraph.....	14
1.	The Enablement Requirement Must Be Applied With Respect to the Claimed Invention.....	14
2.	Appellants Have Affirmatively Demonstrated Compliance With the Enablement Requirement.....	15
3.	Creation of Promoter Fragments is Routine in the Art.....	18
4.	The Examiner Has Failed to Establish a Prima Facie Case of Lack of Enablement	20
D.	The Claims are not anticipated under 35 U.S.C. §102(b).....	22
1.	The Rejection Over McElroy et al	22
2.	The Rejection Over Debonet et al.....	24
VIII.	CLAIMS APPENDIX.....	26
IX.	EVIDENCE APPENDIX.....	28
X.	RELATED PROCEEDINGS APPENDIX.....	29

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Diane M. Ruezinsky

Serial No.: 10/810,788

Filed: March 26, 2004

For: NOVEL PLANT PROMOTERS FOR USE
IN EARLY SEED DEVELOPMENT

Group Art Unit: 1638

Examiner: Page, Brent T.

Atty. Dkt. No.: MONS:060US

BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief. The date for filing this Brief is February 2, 2007. Included herewith is a request for a one month extension of time and the required fees the Appeal Brief and one month extension. Should any additional fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, or should an overpayment be made, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/MONS:060US.

Please date stamp and return the attached postcard as evidence of receipt.

I. REAL PARTY IN INTEREST

The real party in interest is Monsanto Company, the parent company of assignee Monsanto Technology LLC.

II. RELATED APPEALS AND INTERFERENCES

Related appeals are Appeal Nos. 2003-0936 (Serial No. 09/532,806, now U.S. Patent No. 6,747,189) and 2005-0409 (Serial No. 09/757,089, now U.S. Patent No. 7,151,204). Copies of the Board decisions issued in these cases are provided under the Related Proceedings Appendix. While these cases are not related by priority to the current case, they involved many of the same written description and enablement issues presented in the current appeal, share the same Real Party in Interest with the current case, and involved substantially similar issues relative to the current case with respect to promoter fragments. It is therefore believed that these appeals will have a bearing on the current appeal.

III. STATUS OF THE CLAIMS

Claims 1-13 were filed with the application. Claim 11 was canceled during prosecution. Claims 1-10, 12 and 13 are therefore currently pending. Claims 1-10, 12 and 13 were rejected by the Examiner in the Final Action dated July 3, 2006 and are the subject of this appeal. A copy of the appealed claims as they currently stand is included in Section VIII.

IV. STATUS OF AMENDMENTS

An amendment to Claim 1 was filed as provided in 37 C.F.R. §1.116 on November 2, 2006. However, the amendment not entered by the Examiner and thus the amendment is not included in the appealed claims found in Section VIII and not addressed in the arguments herein.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention relates to early seed promoter p63 sequences, isolated from the plant species *Arabidopsis thaliana*, and fragments thereof or sequences with at least 70% identity thereto having promoter activity. Specification at page 2, lines 24-27, page 9, lines 10-28 and page 6, lines 25-34. The claimed invention further relates to transgenic plants and seeds comprising such a promoter sequence. Specification at page 3, lines 10-16. The claimed invention further concerns oil and meal from transgenic plants comprising the promoter sequences and methods for their preparation. Specification at page 3, lines 26-31.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

- (A) Are claims 1-10, 12 and 13 properly rejected as failing to comply with the written description requirement under 35 U.S.C. §112, first paragraph?
- (B) Are claims 1-10, 12 and 13 properly rejected as lacking enablement under 35 U.S.C. §112, first paragraph?
- (C) Are claims 1-10 and 12 properly rejected under 35 U.S.C. §102(b) as being anticipated by McElroy *et al.* (U.S. Patent 6,207,879)?
- (D) Are claims 1-3, 5-7, 10 and 12-13 properly rejected under 35 U.S.C. §102(b) as being anticipated by Debonte *et al.* (U.S. Patent 5,850,026)?

VII. ARGUMENT

A. Substantially Similar Issues Were Decided by the Board in Appeal No. 2003-0936

Appellants note that, prior to the appeal of the current case, substantially similar issues were decided by the Board in favor of the current Real Party in Interest in Appeal No. 2003-0936 (U.S. Ser. No. 09/532,806; “the ‘806 application”). This application was assigned to DEKALB Genetics Corporation, which is a wholly owned subsidiary of the Real Party in Interest,

Monsanto Company. A copy of the previous Decision is attached in the Related Proceedings Appendix below. A subsequent Board decision in another promoter case also presenting these same issues (Appeal No. 2005-0409; U.S. Ser. No. 09/757,089) was subsequently remanded to the Examiner with an order to distinguish the case on the facts or the law from Appeal No. 2003-0936, which the Examiner did not do. This case has now issued.

Appeal No. 2003-0936 concerned written description and enablement rejections made to claims directed to a maize promoter sequence and was handled by the same art unit as the current case. The main independent claim on appeal read as follows:

1. An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.

The main independent claim here reads as follows:

- 1) A promoter comprising an isolated polynucleotide sequence selected from the group of polynucleotide sequences consisting of:
 - a) a polynucleotide sequence comprising the sequence of SEQ ID NO:4;
 - b) a polynucleotide sequence comprising a fragment of the sequence of SEQ ID NO:4;
 - c) a polynucleotide sequence which exhibits a percentage identity of between about 70% identity and 79% identity with the sequence of a) or b);
 - d) a polynucleotide sequence which exhibits a percentage identity of between about 80% identity and 89% identity with the sequence of a) or b) and;
 - e) a polynucleotide sequence which exhibits a percentage identity of between about 90% identity and 99% identity with the sequence of a) or b)
- wherein the promoter is operably linked to a transcribable polynucleotide molecule.

As can be seen, although the wording of the claims is somewhat different, they are substantially similar with respect to element b) in that both cases concern nucleic acid fragments with promoter activity although the claims concern different nucleic acid sequences. Both claims recite a promoter that comprises a fragment of a full length promoter sequence, which was 3536

bp in the case of the '806 application and is 703 bp in the case of the current application. In addition, the current claims also encompass nucleic acid homologs (e.g., comprising from about 70% identity and 79% identity with SEQ ID NO:4) of the full length promoter sequence or fragments thereof. In addition the current claims do not specify a minimum length, although this minimum length is inherent in the limitations of the claims, which are directed to promoter sequences having promoter activity, as described further below.

In response to the final Office Action Appellants submitted a claim amendment that clarified that the percentage identity limitation is intended with respect to the full length sequence of SEQ ID NO:4 rather than fragments thereof, but the Examiner refused to enter the amendment, stating that the term “with promoter function” raised enablement issues. However, as the claim originally rejected was directed to “a promoter...operably linked to a transcribable polynucleotide”, Appellants submit that the refusal to enter the amendment was improper as no new enablement issues were raised and the amendment clearly eliminated this issue on appeal. The amendment should therefore be entered, although the rejection fails with or without entry of the amendment, as set forth below.

In the current case, as with the '806 case previously heard on appeal, the structural definition of claimed fragments is provided in the full length sequence that was included in the application as filed. The teachings of the two applications are also substantively similar, in that both provide extensive guidance as how promoter sequences may be modified (*i.e.*, to generate fragments and structural homologs) and tested for promoter activity. In addition, the knowledge in the art is even more advanced in the current case, given the March 28, 2003 provisional application filing date of the current case relative to the March 21, 2000 filing date of the '806 application. This is particularly significant because the Board already noted in that case that “the

level of skill and knowledge in this art is extremely high” and that “it is well within the ordinary skill of the artisan to determine those nucleotide sequences which are critical for functional [promoter] activity.”

Essentially the same reasoning was applied by the Examiner in Appeal No. 2003-0936 as in this appeal. For example, the Examiner asserted that the claims lacked written description because functional elements within the full length promoter sequence were not specified, *e.g.*, that Appellants did not describe which specific fragments of the full length promoter SEQ ID NO had promoter function. In both cases an enablement rejection was also issued on the basis that, because of the lack of recitation of the specific functional elements, only the full length sequence was enabled.

The Board reversed both rejections by the Examiner. The Board first noted that the claims were supported by a literal written description in the sequence listing. The Board specifically noted the following:

As we understand the rejection, the examiner concedes that appellants’ specification describes every subfragment claimed *which can function as a promoter*. However, that description does not satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, because the subfragments of SEQ ID NO:1 between 95 and 3536 contiguous bases in length that can function as a promoter are not distinguished from the subfragments of SEQ ID NO:1 between 95 and 3536 contiguous bases in length that cannot function as a promoter. The problem with the examiner’s position is that it confuses the written description requirement of 35 U.S.C. § 112, first paragraph, with the enablement requirement of 35 U.S.C. § 112, first paragraph.

(emphasis added) Decision at 2, p. 8.

The Board therefore held that the test for written description applied by the Examiner was not the correct one, and found that the written description requirement was satisfied by the literal description of the sequences in the specification.

The Board then analyzed the enablement rejection by first noting that the field was unpredictable and experimentation would be required to make and use promoter sequences shorter than full length. However, the Board also found that substantial guidance was provided in the specification and the advanced knowledge in the art rendered any experimentation routine and not undue. Decision at p. 22-24. Teachings similar to those pointed to by the Board as supporting enablement are also found in the current application. Specifically, the current application includes the following: detailed teaching regarding the preparation of derivatives of the full length promoter sequence (page 9, line 10 to page 10, line 14); assays of gene expression (e.g., to test the activity of modified promoters sequences) (page 12, line 18 to page 14, line 2); numerous plant transformation methods (page 18, line 20 to page 19, line 7); and production and characterization of stably transformed plants, including methods for assaying for transgene expression (expression level being indicative of promoter activity) (page 19, line 7 to page 19, line 17).

The working examples in particular show the following: isolation of the p63 promoter (Example 1); construction of an expression construct (pMON69812) comprising the p63 promoter (Example 2); transformation and regeneration of fertile transgenic *Arabidopsis* plants comprising p63 promoter expression constructs (Example 3); analysis of reporter gene activity to confirm the extent of developmental stage specific p63 promoter expression (Example 4, Tables 1 and 2); transformation and p63 promoter activity in transgenic soy plants (Example 5); truncation of the p63 promoter to generate the functional p63 promoter fragment, p63tr (SEQ ID NO:4) (Example 6); and expression from the p63tr promoter in the seeds from transformed canola plants (expression observed in 10 out of 10 transformed plants). Further detailed teaching

of the methods for producing and screening promoter constructs is described in the Detailed Description of the Invention as indicated *supra*.

The foregoing guidance is a complete and substantial teaching commensurate with that found enabling in the '806 application. In fact, the '806 application promoter was substantially longer than the currently claimed promoter, and thus more sequence would need to be screened to generate specific fragments with promoter activity. The state of the art is of course even more advanced in the case of the current application, which was filed three years after the '806 application. The Board's decision in the '806 application was therefore made on the same legal issues and under the same substantive factual background. While the previous decision is not binding precedent, there is no basis for this Appeal to be viewed in a manner different than the '806 application. As noted by the Board in the appeal of the aforementioned second application of the Real Party, which specifically cited the decision in the '806 application:

It is a hallmark of our legal system that similar cases are treated similarly. We do not, and no participant in this time honored system may, casually disregard the outcome of cases with similar facts in prior decisions."

Decision at 2, p. 6.

As set forth below, the finding with respect to fragments is equally applicable with respect to promoter sequences with at least 70% identity to SEQ ID NO:4. In the '806 application the claim specifically found enabled and supported by an adequate written description was directed to "An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1." The Board therefore acknowledged that fragments of 95 bp of the full length sequence (3536 bp) were enabled, which is consistent the literature and Appellants' exhibits attached to this Brief. This evidence fully demonstrates that it was routine in the art as of the filing date to delete even substantial portions of promoter sequences while still maintaining promoter activity. In the foregoing case, this included a

truncation of at least 97% ((3536-95)/3536) of the starting promoter. Therefore, that case acknowledged that at least 97% of the starting promoter could be *completely removed* while retaining promoter activity. In the current case, Appellants claim sequences with at least 70% identity to SEQ ID NO:4 (part c), thereby requiring a substantially larger retention of sequences. The written description and enablement of fragments alone is consistent with the previous Board decisions and therefore more than adequately demonstrates that the claims are enabled and supported by an adequate written description, not to mention detailed teachings in the specification and knowledge in the art regarding promoter alterations.

Appellants therefore respectfully request that the same reasoning applied in the '806 and '089 cases be applied in this case and the written description and enablement rejections be reversed accordingly.

B. The Claims Comply with the Written Description Requirement Under 35 U.S.C. §112, First Paragraph

The Examiner rejects claims 1-10, 12 and 13 for failing to comply with the written description requirement of 35 U.S.C. § 112, first paragraph. Specifically, the Examiner alleges that the application only provides written description of the full length promoter sequence comprising SEQ ID NO:4 and does not provide guidance as to which nucleic acid fragments or variants would have promoter function. The rejection is without merit and should be reversed as explained below.

1. Written Description Must Be Analyzed with Respect to the Claimed Invention

The rejection appears to require Appellants to show how or why the claimed promoter sequences function. In particular, the Examiner suggests that Appellants must show which

structural features are necessary for the function of the p63tr promoter and therefore which fragments thereof exhibit promoter function. However, what is relevant under 35 U.S.C. § 112, first paragraph is that Appellants were in possession of the *claimed invention*; and not unclaimed features envisioned in the Action. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991).

The claims are not directed to particular functional elements. For example, claim 1 of the application currently reads as follows:

- 1) A promoter comprising an isolated polynucleotide sequence selected from the group of polynucleotide sequences consisting of:
 - a) a polynucleotide sequence comprising the sequence of SEQ ID NO:4;
 - b) a polynucleotide sequence comprising a fragment of the sequence of SEQ ID NO:4;
 - c) a polynucleotide sequence which exhibits a percentage identity of between about 70% identity and 79% identity with the sequence of a) or b);
 - d) a polynucleotide sequence which exhibits a percentage identity of between about 80% identity and 89% identity with the sequence of a) or b) and;
 - e) a polynucleotide sequence which exhibits a percentage identity of between about 90% identity and 99% identity with the sequence of a) or b)

wherein the promoter is operably linked to a transcribable polynucleotide molecule.

Thus, what is relevant for purposes of written description is that applicants teach the structure (*i.e.*, the nucleic acid sequence) and operability of the p63tr promoter. Appellants have explicitly taught the structure of the p63tr promoter by providing the nucleic acid sequence of SEQ ID NO:4 (a sequence provided with the application as filed). Appellants have also taught that these sequences comprise promoter function, *i.e.*, they may be *operably* linked to a transcribable polynucleotide, for instance see Example 6 of the specification and the tables therein. Appellants

do not lack a written description for what is expressly set forth in the application. While the claims encompass fragments of the sequence of SEQ ID NO:4 and sequences with between about 70% and 79% identity (or between about 80%-89% or 90%-99% identity) with SEQ ID NO:4 or a fragment thereof, these groups define a subset of sequences fully described by SEQ ID NO:4. It is well settled that an applicant need not provide an *ipsis verbis* description for the claimed invention. *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) (stating that the written description requirement does not require an applicant to “describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed” (citations omitted)). Here, the entire scope of claimed subject matter is supported by the literal description in the sequence listing. *The Regents of The University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1568; 43 USPQ2d 1398, 1406 (Fed. Cir. 1997) (noting that a name alone does not satisfy the written description requirement where “it does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, *as one can do with a fully described genus, visualize or recognize the identity of the members of the genus*” (emphasis added)). Thus, the skilled artisan would clearly recognize the claimed genus of *promoters* (i.e., sequences having promoter activity) based upon the structural features (sequences) that are fully described in the specification.

In an attempt to support the rejection the Examiner cites *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ 2d 1016 at 1021, (Fed Cir. 1991) as holding that a gene (as analogized to a promoter here) is not reduced to practice until it can be defined by its “physical and chemical properties.” However, the holding of *Amgen v. Chugai* does not concern the current fact situation and is not relevant to this case since, as described *supra*, the current claims

fully define the genus of promoter sequences based upon their physical and chemical structure, that is as a fragment of SEQ ID NO:4 or sequence with a defined percent of identity of at least about 70% to SEQ ID NO:4. Thus, the invention has been reduced to practice as described in *Amgen v. Chugai*

2. Modification of Promoter Sequences is Routine in the Art

It is well settled that written description must be reviewed from the perspective of one of skill in the art at the time the application is filed. *Wang Labs., Inc. v. Toshiba Corp.*, 993 F.2d 858, 863 (Fed. Cir. 1993). In the current case it was well known as of the filing date that substantial alterations can be made to a promoter sequence while retaining promoter activity and those of skill in the art knew how to make such alterations. For example, Appellants provided **Exhibits A-C** during the prosecution of the case, each of which describe the alteration and modification of promoter sequences. For instance, Welsch *et al.* (**Exhibit A**; “Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana*”; *Planta* 216: 523–534 (2003)) describe the creation of multiple deletion fragments of a plant (*Arabidopsis thaliana*) phytoene synthase gene promoter. Starting with a full length sequence comprising 1746 nucleotides of the 5’ region, truncations were created and tested for expression comprising only 1314, 910, 809, 300 and 196 nucleotides of the 5’ upstream region. Exhibit A at p. 526, FIG. 1. It was shown that the deletion comprising 1314 nucleotides was “almost indistinguishable” in the pattern of expression. *Id.* at p. 526, 2nd col. It was also shown that, while truncation to 196 nucleotides abolished the responsiveness observed in the full length promoter to some types of light, responsiveness (*e.g.*, promoter activity) was still observed to several types of light. *Id.* Therefore, the authors showed that at least 432/1746 (*e.g.*, 24%) nucleotides could be deleted from the full length promoter with essentially *no* change in

expression and at least 1550/1746 (*e.g.*, 89%) nucleotides could be deleted while still retaining promoter activity.

Similarly, Piechulla *et al.* (**Exhibit B**; “Identification of tomato Lhc promoter regions necessary for circadian expression” *Plant Molecular Biology* 38: 655–662, 1998) describe the deletion analysis of promoters from the cab 1A, cab 1B, cab 8 and cab 11 genes from the tomato light harvesting complex of genes to determine which deletions would affect circadian expression. Deletion constructs were tested comprising from between 1091 and 43, from between 793 and 152, from between 322 and 148 and from between 251 to 119 nucleotides from these promoters, respectively. **Exhibit B**, p. 659, Fig. 5. As explained by the authors, the “results show that the short 5'-upstream regions are sufficient for a basal mRNA accumulation” and further indicate that upstream sequences are responsible for circadian rhythm. The results therefore indicate that at least 1048/1091 (*e.g.*, 96%), 641/793 (*e.g.*, 81%), 174/322 (*e.g.*, 54%) and 132/251 (*e.g.*, 53%) of the nucleotides of these promoters could be deleted while still retaining promoter activity.

The foregoing is also illustrated by other studies, such as that of Cho and Cosgrove (**Exhibit C**; “Regulation of root hair initiation and expansin gene expression in Arabidopsis,” *Plant Cell*, 14, 3237–3253, 2002). These authors showed that more than 990 base pairs of an approximately 1428 bp plant promoter sequence designated AtEXP7 could be deleted without significantly affecting promoter activity and even larger deletions could be made while maintaining a reduced promoter activity. See **Exhibit C**, p. 3244, 2nd col. and FIG. 8. It was also shown that a deletion of approximately 775 bp could be made from a 1058 bp plant promoter designated AtEXP18 without significantly reducing promoter activity. See *Id.* at FIG. 10. Finally, the authors further showed that numerous substitution mutations could be made in a

fragment of AtEXP7, while retaining full promoter activity and in some cases increasing activity. See. *Id.* at FIG. 9 and p. 3245, 2nd col. These studies therefore show that fragments of full length promoter sequences can routinely be made that retain promoter activity.

The foregoing therefore demonstrates that Appellants were in possession of the invention. Appellants need not show how or why the claimed promoter sequences function, for example, by specifying which functional elements cause promoter activity. What is relevant under 35 U.S.C. § 112, first paragraph, is that the applicants were in possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991). The claims are not directed to particular functional elements, but rather to nucleic acids having promoter function. Written description for the claimed invention, is therefore present based on the description of SEQ ID NO:4 and accompanying detailed descriptions and teachings in the specification when properly viewed in the context of the knowledge in the prior art, as required under Federal Circuit precedent.

All of the claimed subject matter has therefore been fully described pursuant to 35 U.S.C. § 112, first paragraph. Reversal of the rejection is thus respectfully requested.

C. The Claims Are Enabled Under 35 U.S.C. §112, First Paragraph

The Examiner rejects claims 1-10, 12 and 13 as not being enabled by the specification. In particular, the Action alleges that the specification is enabling only for the entire 703 nucleotide sequence of SEQ ID NO:4. The rejection should be reversed as explained below.

1. The Enablement Requirement Must Be Applied With Respect to the Claimed Invention

The Examiner alleges that the specification does not disclose certain structural and functional information regarding which fragments derived from or homologous to SEQ ID NO:4

would have promoter function. However, Appellants note that which structural or functional elements are present is irrelevant. Appellants need not describe why or how the invention works. All that is required under 35 U.S.C. §112, first paragraph, is that the specification teaches one reasonably skilled in the art how to make and use the claimed invention without undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That is, Appellants must only enable what is claimed. See *Durel Corp. v. Osram Sylvania Inc.*, 256 F.3d 1298, 1306-07 (Fed. Cir. 2001). Here, the claimed invention represents fragments or close structural homologs to SEQ ID NO:4 that comprise *promoter activity*, the use thereof and compositions produced there from. As described below, the specification has fully enabled this subject matter.

2. Appellants Have Affirmatively Demonstrated Compliance With the Enablement Requirement

The current claims recite nucleic acids comprising fragments or close structural homologs of SEQ ID NO:4 with promoter activity. Provided in the Sequence Listing of the specification is the nucleic acid sequence of SEQ ID NO:4. This is more than adequate to fully enable one of skill in the art to prepare nucleic acid sequences that are, for example, about 70% identical to SEQ ID NO:4 or that are a fragment of SEQ ID NO:4 or homologous to such a fragment. The Examiner, however, suggests that one of skill in the art would be without guidance in obtaining the claimed fragments of this sequence because the specification does not provide guidance regarding functional promoter regions derived from SEQ ID NO:4. However, the structural information of this subject matter is the sequence itself, which is given in SEQ ID NO:4.

The Examiner further ignores extensive teaching in the specification that go well beyond what is required under the first paragraph of 35 U.S.C. § 112. For example, provided in the

specification from page 6, line 25-34 and on page 9, line 10 to page 10, line 14, is detailed teaching regarding the preparation of derivatives of the full length promoter sequence. Described from page 12, line 18, to page 14, line 2, of the specification are plant reporter constructs and methods for using the same to determine the activity of a promoter sequence. In particular, on page 13, line 1-9 methods are described for using scorable or screenable markers to “evaluate the potential expression profile of the promoters or promoter fragments when operatively linked to genes.” Additionally plant transformation and culture methods are provided on page 18, line 20 to page 19, line 17. Methods for making and using plant parts that comprise nucleic acid sequences of the invention are also described in detail on page 19, line 18 to page 21, line 17.

In the working examples, Example 1 at page 26 describes the isolation of the *Arabidopsis* p63 promoter from plant genomic sequence using a PCR-based strategy. The Example provides the precise sequence of the primers used for such isolation. The sequence of the isolated p63 promoter was provided in the application as filed as SEQ ID NO:3, which includes the p63tr sequence of SEQ ID NO:4. Example 2 at page 28 describes the construction of reporter constructs comprising the *uidA* (GUS) reporter gene positioned downstream of the p63 promoter. The methods used for accessing promoter activity in transformed plants by determining the amount of GUS expression are also detailed.

Providing yet further detail, Example 5 teaches the construction of a further p63 GUS expression vector and its transformation into soy via *Agrobacterium* introduction.

Example 6 teaches the construction of an expression vector with a fragment of the p63 promoter, p63tr. Also, provided are further methods for assessing promoter expression in transformed plants (*i.e.*, in seeds from canola plants). Importantly, this example provides

detailed disclosure of methods for making and testing expression vectors comprising plant promoter fragments. Additional disclosure of these methods is provided in the Detailed Description as described *supra* (pages 12-14 of the specification).

The foregoing teachings in Appellants' specification are more than adequate to enable the full scope of the invention and cannot properly be ignored. *In re Wands*, 858 F.2d at 737. It would be a straightforward matter for one of skill in the art to identify fragments of SEQ ID NO:4 having promoter activity, especially given the detailed teachings in the specification. While Appellants acknowledge this would require some routine screening, "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands*, 858 F.2d at 737. Furthermore, some amount of experimentation is permissible, especially when the specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Id.* (quoting *Ex parte Jackson*, 217 USPQ 2d 804, 807 (Bd. App. 1982)). The detailed teaching in the specification has provided all of the methodology necessary for creating and screening the subject fragments (or homologs) for promoter activity.

Given the fact that SEQ ID NO:4 is only 703 nucleic acids long, the Examiner's assertion that it would require undue experimentation to create fragments of the sequence and screen them for activity could not be further from the truth. This is underscored by the fact that the working examples provide exactly the methodology that one could use to make fragments and screen them for activity. Specifically, Example 6 describes inserting a p63 promoter fragment into a transformation construct, transforming recipient cells with the constructs and screening the subsequently derived transgenic plants for activity. These teaching fully demonstrate the enablement of the claims.

3. Creation of Promoter Fragments is Routine in the Art

Appellants further note that the teaching in the specification and numerous other art references demonstrate that it was routine in the art as of the application filing date to make and test promoter fragments and variants for activity. As evidence of the state of the art at the time the application was filed, Appellants provided during prosecution **Exhibits A-C**, each of which describe the alteration and modification of promoter sequences. For example, Welsch *et al.* (**Exhibit A**) describe the creation of multiple deletion fragments of an *Arabidopsis thaliana* phytoene synthase (psy) gene promoter. Welsch generated multiple promoter fragments of various lengths (*i.e.*, 1314, 910, 809, 300 and 196 nucleotides) and tested each for activity. **Exhibit A** at p. 526, FIG. 1. The studies described in Welsch demonstrated that approximately 89% of a starting promoter sequence could be deleted while still retaining promoter activity and at least 24% could be deleted without appreciably altering activity. Similarly, Piechulla *et al.* (**Exhibit B**) describes the deletion analysis of promoters from the cab 1A, cab 1B, cab 8 and cab 11 genes from the tomato light harvesting complex of genes to determine which deletions would affect circadian expression. Promoter deletion constructs constructed and tested comprise from between 1091 and 43, from between 793 and 152, from between 322 and 148 and from between 251 to 119 nucleotides from the full-length promoters. **Exhibit B**, p. 659, Fig. 5. Results presented in Piechulla *et al.* showed that approximately 96%, 81%, 54% and 53% of four promoter sequences analyzed could be deleted while still retaining promoter activity, even if decreased or somewhat altered. The state of the art is further demonstrated by Cho and Cosgrove (**Exhibit C**). These authors made numerous deletion fragments and nucleotide substitutions in two plant promoters including the 1428 bp sequence designated AtEXP7. Each of the AtEXP7 promoter fragments and homologs (*i.e.*, promoters comprising nucleotide substitutions) was tested for promoter activity (**Exhibit C**, pgs. 3246-3247, Figs. 8 and 9). The

studies in Cho and Cosgrove demonstrated that more than 990 base pairs of the 1428 bp promoter could be deleted without significantly effecting promoter activity and even larger deletions could be made while maintaining a reduced promoter activity. Over all, Cho and Cosgrove showed that at least 69% and 73% of two promoters analyzed could be deleted without significantly affecting promoter activity, and further that numerous substitution mutations could be made while still retaining full promoter activity (and in some cases increasing promoter activity). **Exhibit C**, p. 3244, 2nd col., FIGs. 8-10, p. 3245, 2nd col. Each of the foregoing studies show that fragments and structural homologs of full length promoter sequences can routinely be made tested for promoter activity.

It would therefore be a straightforward matter for one of skill in the art to generate sub fragments of SEQ ID NO:4 and 70% identical sequences that retain promoter activity, especially given the detailed teachings in the specification and knowledge in the art. While this would require some routine screening, “[e]nablement is not precluded by the necessity for some experimentation such as routine screening.” *In re Wands*, 858 F.2d at 737. Where the specification “provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed” this does not constitute undue experimentation. *Id.* (quoting *Ex parte Jackson*, 217 USPQ2d 804, 807 (Bd. App. 1982)). This is underscored by the fact that those of skill in the art knew how to make such routine changes and assess their effect on activity as of filing date. Therefore, given the detailed teaching in the specification, advanced state of the art and scope of claimed subject matter, compliance with the enablement requirement has been fully demonstrated.

4. The Examiner Has Failed to Establish a *Prima Facie* Case of Lack of Enablement

The Examiner has provided no basis to doubt the enablement of the instant claims. The Examiner presents two references and argues that they support the rejection by demonstrating that certain deletions and mutations in promoter sequences may reduce or abrogate promoter activity. Specifically the Examiner cites Kim *et al.* (**Exhibit D**, *Plant Mol. Biol.*, 24:105-117, 1994) and Dolferus *et al.* (**Exhibit E**, *Plant Physiology*, 105:1075-1087, 1994) each of which concerns studies of plant promoter activity and show that certain mutations and deletions reduce promoter activity. Appellants fully acknowledge that some deletions and mutations will reduce the activity of a promoter. However, contrary to the Examiners assertions, this fact and the references cited in no way cast doubt on enablement and the references in fact *demonstrate* enablement of the instant claims.

Both Kim and Dolferus employ standard screening methods to assess the promoter activity of sequences comprising deletions and/or point mutations. For instance, in Kim *et al.*, the authors demonstrate that numerous mutations can be made even in the so called critical promoter region focused on between position -131 and -112. Kim *et al.*, explained that low level promoter activity was still obtained “even when the entire hexamer sequence was removed (oligomer 125-112).” Kim *et al.* at p. 109, 1st col, bottom ¶. Table 2 further shows numerous point mutations that were created, all but two of which still retained promoter function. It must further be noted that Kim *et al.* specifically focused on the regions of the promoter that were identified by the authors as the *most critical* to function and sought out those deletion fragments that had no activity. Kim *et al.* therefore shows that as of at least the 1993 publication date those of skill in the art could make substantial modifications to the most critical regions of a promoter while still retaining promoter function. This reference therefore more than adequately

demonstrates that as of the filing date it was *routine for those of skill in the art* to create promoter deletion and mutation fragments retaining promoter activity.

Turning to the Dolferus reference, the authors show that deletion of a section from -964 to -510 of the 1kb (-964 to +53) promoter region of the Adh gene “results in *increased expression* under uninduced and all stress conditions, suggesting that this region contains a repressor binding site.” (emphasis added) See Abstract of Dolferus *et al.* Therefore, this alone shows that well over 30% of the promoter could be deleted altogether while obtaining an even more effective promoter element. The reference therefore again established enablement by showing that as of at least **1994** it was routine in the art to mutate and delete over 30% of a promoter while still retaining promoter function. Thus, like the Welsch, Piechulla and Cho references, both Kim and Dolferus provide yet further evidence that only routine experimentation is required to determine whether a promoter fragment or homolog comprises promoter activity. While some sequences may be necessary for promoter function, the identification of those sequences is routine.

Furthermore, the Examiner has failed to set forth a case that undue experimentation would be required to practice the invention across the full scope of the instant claims. It is the Examiner that bears the burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by a claim is not adequately enabled by the description of the invention in the specification. *In re Wright*, 9 U.S.P.Q.2d 1510, 1512-1513 (Fed. Cir. 1993) (citing *In re Marzocchi*, 169 U.S.P.Q. 367, 369-70 (CCPA 1971)). Thus, the evidence presented above in the form of at least five different published reports cited by Appellants or the Examiner demonstrate that one of skill in the art, in view of the teaching in the specification, could readily have made and used the claimed sequences without undue experimentation. A statement

doubting the enablement of an Appellants' claims without providing an objective basis does not meet the meet the standard set forth in *Marzocchi*, "[o]therwise, there would be no need for the Appellant to go to the trouble and expense of supporting his presumptively accurate disclosure." *In re Marzocchi*, 169 U.S.P.Q. at 370. Thus, without more, the rejection must fail.

It is finally noted that the legal standard for enablement does not require that Appellants demonstrate enablement for all possible claimed iterations. Enablement must bear only a reasonable relationship to the scope of the claims. *In re Fisher*, 166 U.S.P.Q. 18, 24 (CCPA 1970). For example, a patent applicant is not required to "predict every possible variation, improvement or commercial embodiment of his invention." *United States Steel Corp. v. Phillips Petroleum Co.*, 673 F. Supp. 1278, 1292 (D. Del. 1987), *aff'd*, 865 F.2d 1247, 1250 (Fed. Cir. 1989) (specifically quoting this statement). This is echoed in the MPEP: "[a]s long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied." MPEP 2164.01(b) (citing *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (CCPA 1970)).

In view of the foregoing, Appellants respectfully submit that the full scope of the claims has been enabled. Reversal of the rejection under 35 U.S.C. §112, first paragraph for lack of enablement is thus respectfully requested.

D. The Claims are not anticipated under 35 U.S.C. §102(b)

1. The Rejection Over McElroy *et al.*

The Examiner rejects claims 1-10 and 12 under 25 U.S.C. § 102(b) as allegedly anticipated by McElroy *et al.* (U.S. Patent 6,207,879). Specifically, the Examiner has alleged that the promoter sequences of claim 1 encompass the RS81 promoter that is described in

McElroy. As previously noted by Appellants and as acknowledged by the Examiner, SEQ ID NO:4 has *no significant homology* to the RS81 promoter, SEQ ID NO:1 of U.S. Patent 6,207,879. This fact is clearly shown by the sequence alignment that was previously made of record in the case. Nonetheless, the Examiner asserts that a fragment of SEQ ID NO:4 as recited in claim 1 reads on a one-nucleotide sequence. However, the current claims require a *promoter sequence* and a single nucleotide is not a promoter. Thus, the Examiner's assertions are without any factual support and do not constitute a *prima facie* case for anticipation.

What constitutes a promoter is known in the art, and this term is defined in the specification on page 5, lines 16-19, as: "a polynucleotide molecule that in its native state is located upstream or 5' to a translational start codon of an open reading frame (or protein coding region) and that is involved in recognition and binding of RNA polymerase II and other proteins (trans-acting transcription factors) *to initiate transcription*" (emphasis added). Contrary to the assertions made by the Examiner, a single nucleotide would not be capable of initiating transcription and, in any case, no evidence has been provided on the record to support the Examiner's arguments. In fact, this is confirmed by each of **Exhibits A-C**, which describe deletion studies carried out to identify minimal active promoter sequences. Furthermore, claim 1 states that "the promoter is *operably* linked to a transcribable polynucleotide molecule." As taught in the instant specification "a promoter is operably linked to a gene of interest if the promoter regulates or mediates transcription of the gene of interest in a cell," (page 11, lines 6-8). The Examiner has provided no evidence to demonstrate that a single nucleotide sequence would be sufficient to mediate transcription of a gene in a cell. Thus, such a sequence could hardly be operably linked to a transcribable polynucleotide molecule. The references attached as **Exhibits A-C** and those cited and relied upon by the Examiner (**Exhibits D-E**) each show that,

while substantial modifications can be made to a promoter element, a minimal promoter sequence is needed for activity that is well more than sufficient in size to distinguish the claimed promoter sequence. Certainly in no instance does any evidence of record show that a single nucleotide or any minimal segment shared between the cited and claimed sequences would be sufficient for promoter activity. Thus, there is no factual basis on the record to support the Examiner's rejection over McElroy. Reversal of the rejection is therefore respectfully requested.

2. The Rejection Over Debonte *et al.*

The Examiner also rejects claims 1-3, 5-7, 10 and 12-13 under 25 U.S.C. § 102(b) as allegedly anticipated by Debonte *et al.* (U.S. Patent 5,850,026). Once again, Debonte does not disclose any sequences that exhibit any reasonable degree of homology to SEQ ID NO:4 a fact not disputed by the Examiner. Nonetheless, the Examiner again argues that the instant claims read on a single nucleotide and thus the claims are anticipated by the sequences described in Debonte. As stated in detail *supra* a single nucleotide does not constitute a fragment of SEQ ID NO:4 that may be operably linked to a gene. Thus, like McElroy, Debonte fails to disclose all of the elements of the instant claims and therefore it can not anticipate the instant claims.

Reversal of these rejections under 35 U.S.C. § 102(b) is thus respectfully requested.

CONCLUSION

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

/Robert E. Hanson/

Robert E. Hanson
Reg. No. 42,628
Attorney for Appellants

FULBRIGHT & JAWORSKI L.L.P
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 536-3085

Date: February 2, 2007

VIII. CLAIMS APPENDIX

APPEALED CLAIMS:

1) (Previously presented) A promoter comprising an isolated polynucleotide sequence selected from the group of polynucleotide sequences consisting of:

a) a polynucleotide sequence comprising the sequence of SEQ ID NO:4;

b) a polynucleotide sequence comprising a fragment of the sequence of SEQ ID NO:4;

c) a polynucleotide sequence which exhibits a percentage identity of between about 70% identity and 79% identity with the sequence of a) or b);

d) a polynucleotide sequence which exhibits a percentage identity of between about 80% identity and 89% identity with the sequence of a) or b) and;

e) a polynucleotide sequence which exhibits a percentage identity of between about 90% identity and 99% identity with the sequence of a) or b)

wherein the promoter is operably linked to a transcribable polynucleotide molecule.

2) (Original) A construct comprising the promoter of claim 1, wherein said promoter is operably linked to a transcribable polynucleotide molecule operably linked to a 3' transcription termination polynucleotide molecule.

3) (Original) The construct of claim 2, wherein said transcribable polynucleotide molecule is a gene of agronomic interest.

4) (Original) The construct of claim 2, wherein said transcribable polynucleotide molecule is a marker gene.

5) (Original) A transgenic, seed-producing dicotyledonous plant stably transformed with a construct comprising the promoter of claim 1, wherein said promoter is operably linked to a transcribable polynucleotide molecule operably linked to a 3' transcription termination polynucleotide molecule.

- 6) (Original) The transgenic dicotyledonous plant of claim 5, wherein said plant is a dicotyledonous plant selected from the group consisting of tobacco, tomato, potato, peanut, soybean, cotton, canola, rapeseed, safflower, flax, sugarbeet, *Arabidopsis*, Brassica, sunflower, and alfalfa.
- 7) (Original) The transgenic dicotyledonous plant of claim 5, wherein said transcribable polynucleotide molecule confers altered oil content in the seed to said transgenic plant.
- 8) (Original) The transgenic dicotyledonous plant of claim 5, wherein said transcribable polynucleotide molecule confers altered protein quality in the seed to said transgenic plant.
- 9) (Original) The transgenic dicotyledonous plant of claim 5, wherein said transcribable polynucleotide molecule confers altered micronutrient content in the seed to said transgenic plant.
- 10) (Currently amended) A seed of said transgenic plant of claim 5, wherein the seed comprises said construct.
- 11) (Canceled)
- 12) (Original) Meal from said transgenic plant of claim 5.
- 13) (Original) A method of making a vegetable oil and meal, comprising the steps of: a) incorporating in the genome of a dicotyledonous seed producing, oil-containing plant a promoter according to claim 1 operably linked to a transcribable polynucleotide molecule conferring altered oil content; b) growing the dicotyledonous plant to produce seeds; and c) extracting oil from the seed to produce extracted oil and meal.

IX. EVIDENCE APPENDIX

- Exhibit A:** Welsch *et al.*, “Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana*” *Planta* 216:523–534, 2003. Entered with Applicants Response to Office Action.
- Exhibit B:** Piechulla *et al.*, “Identification of tomato Lhc promoter regions necessary for circadian expression” *Plant Molecular Biology* 38: 655–662, 1998. Entered with Applicants Response to Office Action.
- Exhibit C:** Cho and Cosgrove, “Regulation of root hair initiation and expansin gene expression in *Arabidopsis*,” *Plant Cell*, 14, 3237–3253, 2002. Entered with Applicants Response to Office Action.
- Exhibit D:** Kim *et al.*, “A 20 nucleotide upstream element is essential for the nopaline synthase (nos) promoter activity,” *Plant Mol. Biol.*, 24:105-117, 1994. Cited by Examiner.
- Exhibit E:** Dolferus *et al.*, “Differential interactions of promoter stress responses of the *Arabidopsis* elements in Adh Gene,” *Plant Physiology*, 105:1075-1087, 1994. Cited by Examiner.

X. RELATED PROCEEDINGS APPENDIX

**Decision in Appeal No. 2003-0936 (U.S. Ser. No. 09/532,806, now U.S. Patent No. 6,747,189)
mailed August 29, 2003**

**Order in Appeal No. 2005-0409 (U.S. Ser. No. 09/757,089, now U.S. Patent No. 7,151,204)
mailed April 29, 2005**

EXHIBIT A

Ralf Welsch · Joaquín Medina · Giovanni Giuliano
Peter Beyer · Johannes von Lintig

Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana*

Received: 21 March 2002 / Accepted: 15 July 2002 / Published online: 18 September 2002
© Springer-Verlag 2002

Abstract The expression of the gene coding for the carotenogenic enzyme phytoene synthase is highly regulated. To study this, its promoter and truncated versions thereof were translationally fused to the luciferase gene as a reporter and these constructs were used to transform *Arabidopsis thaliana*. The full-length promoter was shown to be active in the dark, but mediated positive responses towards different light qualities (far-red, red, blue and white light). Among the herbicides tested, norflurazon and gabacluline showed no notable effects, while CPTA abolished light induction completely. Response towards different light qualities was mediated by a TATA box-proximal promoter region up to position –300, containing G-box-like elements involved in the distinction of different monochromatic light qualities applied. This is detected in electrophoretic mobility shift assays (EMSAs), which reveal differential complex formation. A TATA box distal region of the promoter was shown to be responsible for a high basal promoter activity that was not modulated by different light qualities. Using EMSAs, a novel *cis*-acting element ATCTA occurring in tandem between positions –854 and –841 proved to be decisive in this respect. The motif was found in several other promoter regions involved in carotenoid and tocopherol biosynthesis, as well as in the

promoter regions mediating the expression of photosynthesis-related genes. The functional equivalence of the motifs was shown by successfully using the respective regions in EMSAs. We conclude that the ATCTA motif represents an element capable of mediating a coordinated regulation of these pathways at the transcriptional level.

Keywords Carotenoid · Light regulation · Norflurazon · Phytochrome · Phytoene synthase

Abbreviations CPTA: 2-(*p*-chlorophenylthio)triethylammonium chloride · GBF: G-box binding factor · GGPS: geranylgeranyldiphosphate synthase · EMSA: electrophoretic mobility shift assay · PDS: phytoene desaturase · PHY: phytochrome · PSY: phytoene synthase

Introduction

The biosynthesis of carotenoids is regulated in response to both developmental and environmental stimuli, e.g. during chromoplast development in flowers and fruits or during the process of chloroplast development. In chromoplasts, carotenoids are massively accumulated to exert mainly ecological functions, while in chloroplasts a coordinated supply of carotenoids and chlorophylls is physiologically crucial. Carotenoid-free plants cannot survive in the light because in the photosynthetic apparatus carotenoids function in both the acquisition of light energy and the protection against light (Demmig-Adams et al. 1996). To perform these tasks, these pigments are localized together with chlorophyll molecules in the reaction centers of the photosystems as well as in the light-harvesting complexes.

Among several environmental stimuli regulating chloroplast development, light is the most important. Here, light is perceived differentially, e.g. by the phytochrome system and by cryptochromes, to control this process (for reviews, see Frankhauser and Chory 1997;

R. Welsch · J. Medina · P. Beyer · J. von Lintig (✉)
Center for Applied Biosciences, Universität Freiburg,
Schänzlestrasse 1, 79104 Freiburg, Germany
E-mail: lintig@uni-freiburg.de
Fax: +49-761-2032926

Present address: J. Medina
INIA, Edificio Z, Ctra Coruña Km7,
2040 Madrid, Spain

Present address: G. Giuliano
Casaccia Research Center, Via Anguillarese 301,
00060 S.M. di Galeria, Roma, Italy

Present address: J. von Lintig
Institut für Biologie I,
Hauptstrasse 1, 79104 Freiburg, Germany

Batschauer 1998, Lin 2000). Since light plays a dual role and may exert deleterious effects on the emerging photosynthetic apparatus, there is a requirement for mechanisms ensuring a protective function against light, as reflected by the quantitatively and qualitatively coordinated biosynthesis of carotenoids and chlorophylls. Accordingly, the expression of carotenogenic genes during de-etiolation must be precisely regulated and coordinated with the expression of genes for carotenoid-bearing protein complexes involved in photosynthesis, as well as with the expression of genes involved in chlorophyll biosynthesis, etc.

We investigated previously the expression of carotenogenic mRNAs during de-etiolation using quantitative RT-PCR. This revealed that transcripts of geranylgeranylphosphate synthase (*ggps*) and phytoene desaturase (*pds*) remained relatively constant while a significant up-regulation of phytoene synthase (*psy*) was observed during this process. It was concluded that the up-regulation of carotenoid biosynthesis relied essentially on the transcriptional activation of the *psy* gene, which codes for the first enzyme specifically committed to carotenoid biosynthesis. A detailed analysis of the light qualities mediating the light induction of *psy* revealed that the phytochrome system was involved. The responses of *phyA* and *phyB* mutants allowed us to assign a major role to PHYA; in contrast, PHYB was not involved. However, since in *phyA/phyB* double mutants increased *psy* transcript amounts were observed under red (R) and blue (B) light, the involvement of other phytochromes (in *Arabidopsis thaliana* PHYC to PHYE) and of cryptochromes could not be excluded (von Lintig et al. 1997). In an extension of these studies, it was shown later in *Sinapis alba* that posttranscriptional as well as posttranslational events, such as the formation of the prolamellar body and of competent membrane structures, are decisive in the regulation of the enzymatic activity of PSY and thus of carotenoid biosynthesis (Welsch et al. 2000).

The corresponding situation in chromoplast-bearing plants may be more complex. Taking tomato fruit as an example, the formation of large amounts of carotenoids involves at least in part a second set of genes differing in the tissue specificity of their expression. Two differentially regulated *psy* genes (*psy1* and *psy2*) as well as two lycopene β -cyclase (*cyc-b* and *lcy-b*) genes exist here. *Psy1* plays a predominant role in the non-green tissue of fruit beyond the breaker stage, while *psy2* is involved in the carotenoid biosynthesis of green leaves. Similarly, *cyc-b* and *lcy-b* are predominantly involved in carotenoid biosynthesis in chromoplast or chloroplast-bearing tissues, respectively (for recent reviews, see van den Berg et al. 2000; Hirschberg 2001). The transcriptional regulation of the carotenoid biosynthesis genes seems light-regulated via the phytochrome system in tomato fruit (Alba et al. 2000), but the coordination with genes involved in photosynthesis, which is very strict in leaves, is lost. Thus hitherto unknown developmental factors must be involved. One further determinant of carotenoid

accumulation in chromoplasts is provided by the formation of carotenoid sequestering structures such as lipid globules, crystals, membranes or proteolipid fibrils (Rabbani et al. 1998; for review see Camara et al. 1995).

To circumvent the complications given above, we selected *A. thaliana* as a system to investigate the regulation of carotenoid biosynthesis in photosynthetically active tissues. *A. thaliana* does not develop chromoplasts and *psy* is present as a single copy gene.

In the present investigation, we report on the cloning and the structural and functional analysis of the *psy* promoter to substantiate its key regulatory role in green tissues. Using transgenic *A. thaliana* lines transformed with the promoter fused to the luciferase gene as a reporter, we investigated promoter activity in different tissues, under different light qualities and in the presence of herbicides. The use of promoter truncations in combination with gel retardation assays to detect DNA-complex formation allowed the characterization of responsible *cis*-acting elements. Our results indicate a spatial separation of *cis*-acting elements mediating different light responses, as well as the existence of a novel *cis*-acting element enabling strong basal activity. This latter element was also found in a variety of different photosynthesis-related genes, indicating a possible co-regulation at the transcriptional level.

Materials and methods

Cloning of the *psy* gene

For the isolation of the *psy* gene, a genomic library of *Arabidopsis thaliana*, ecotype Wassilewskija (Schulz et al. 1994) was screened. As a probe, a PCR fragment was amplified from a plasmid carrying the *A. thaliana psy*-cDNA (GenBank accession number L25812; Bartley and Scolnik 1994) with the oligonucleotides 5'-TTG TGG GTT GGT AAG GGT TC-3' and 5'-CGT AGA TTG CCC AAA TCG CC-3' and radiolabelled using the Klenow fragment (Klenow and Henningsen 1970). From the isolated genomic clone, a 7-kb *EcoRI* fragment containing the *psy* gene was subcloned into pBSK. 5' subclones were produced using restriction sites and the exonuclease method with the enzymes *Bam*HI and *Pst*I (Henikoff 1984) and then sequenced.

Translational fusions of different promoter truncations with the luciferase gene were produced by subcloning corresponding promoter fragments into the vector pSPLuc⁺ (Promega, Mannheim, Germany). For binary constructs, *psy/luc* fusions were isolated and subcloned into the vector pBIN-35S-mGFP (kindly provided by Dr. M. Rodriguez), replacing the 35S-mGFP region.

Luciferase and GUS measurement

Luciferase activity in seedlings from homozygous T2 lines was determined according to Iida et al. (1995) using a luminometer (lumet LB9501, Berthold, Wildbad, Germany). Protein concentration in the supernatants was determined according to Bradford (1976). All measurements were repeated three times for each T2 line; the values represent the average of all experiments for at least two strongly expressing lines of each transformation.

Quantification of transient expression was performed according to Norris et al. (1993) using the plasmid pHG35SGUS (kindly provided by Dr. M. Rodriguez) as control plasmid. Leaves of 3-week-old *Arabidopsis* plants were bombarded with 14 pmol of an equimolar mixture of control plasmid and test plasmid (i.e. *psy/luc* fusions in the vector pSPLuc⁺) using a self-made particle influence

gun (Feiner et al. 1992). After incubation for 24 h in dim light, extracts were prepared and protein concentration (Bradford 1976) and luciferase activity (Iida et al. 1995) were determined immediately. Remaining extracts were kept for 24 h at -80°C , re-centrifuged and GUS activity was measured using a fluorescence spectrometer (LS50B, Perkin Elmer, Rodgau-Jügesheim, Germany).

Electrophoretic mobility shift assay

Nuclear extracts were isolated from cotyledons of mustard seedlings according to Dignam et al. (1983) and Jensen et al. (1988). Protein concentration was performed according to Bradford (1976) and aliquots were stored at -80°C .

DNA fragments used as probes which were larger than 30 bp were end-labeled with $\alpha\text{-}^{32}\text{P}$ dATP or $\alpha\text{-}^{32}\text{P}$ dCTP. Smaller probes were prepared using partially overlapping oligonucleotides and filled-in with $\alpha\text{-}^{32}\text{P}$ dATP or $\alpha\text{-}^{32}\text{P}$ dCTP using the Klenow fragment (Klenow and Henningsen 1970). DNA fragments used as competitors were prepared by the identical procedure except that all nucleotides were supplied non-radioactive. All DNA fragments were purified by gel chromatography (MicroSpin S-200 or G-50, Amersham Pharmacia Biotech, Freiburg, Germany) and incorporation of radioactivity was quantified by scintillation counting.

Binding reactions were carried out in a final volume of 30 μl and contained 15 μl $2\times$ binding buffer [24 mM Tris/HCl pH 7.9; 24% glycerol (v/v); 70 mM KCl; 0.14 mM EDTA; 0.95 mM PMSF, 2.15 mM DTT; 15 mM MgCl_2 ; 0.01% bromophenol blue (w/v)], 6–10 nmol probe, 2 μg poly(dIdC).poly(dIdC) (Boehringer, Mannheim, Germany), 2 μg nuclear proteins and specific competitor DNA as indicated in the figure legends. After incubation at room temperature for 10 min, the binding mixtures were loaded on 4–8% polyacrylamide gels and run in 25 mM Tris/HCl pH 8.3, 190 mM glycine and 1 mM EDTA at 4°C and 200 V. Gels were wrapped in cellophane, dried at 60°C under vacuum and autoradiographed.

Plant transformation and growth conditions

A. thaliana (ecotype Wassilewskaja; Arabidopsis Biological Resource Center, The Ohio State University, Columbus, Ohio, USA) plants were transformed by vacuum infiltration (Bechtold et al. 1993) with agrobacteria strain GV3101 (Koncz and Schell 1986) containing the binary *psy* promoter/luciferase constructs (see above). Putative T1 transformants were selected on kanamycin-containing (50 $\mu\text{g ml}^{-1}$) Murashige-Skoog (MS) agar plates (1 \times MS salts; 0.5 g MES/KOH, pH 5.7; 0.4% phytoagar). Of the initially identified T1 transformants, 5 lines per transformation were propagated. Homozygous T2 progenies were identified by the selection pattern of the corresponding T3 progenies on kanamycin-containing MS agar plates.

Seeds of white mustard (*Sinapis alba* L., harvest 1982) used for isolation of nuclear extracts were obtained from Asgrow Company (Freiburg, Germany). Seeds from homozygous *Arabidopsis* lines were surface-sterilized, plated on MS agar plates, vernalized for 4 days at 4°C and germinated for 3 days in darkness. Mustard seedlings were germinated and grown for 3 days on moist paper in darkness. Subsequently, seedlings were illuminated for 24 h with the following light conditions: white light (W), Osram L40 W (73 lamps) + Philips TLD40 W (18 lamps), fluence rate 10.9 W m^{-2} , red light (R), $\lambda_{\text{max}} = 660$ nm, fluence rate 5 W m^{-2} ; far-red (FR), $\lambda_{\text{max}} = 730$ nm, fluence rate 3 W m^{-2} ; blue light (B), Philips TLD36 W/18 lamps, plexiglass filter 627 (Röhrl & Haas, Darmstadt, Germany), $\lambda_{\text{max}} = 436$ nm, fluence rate 4.1 W m^{-2} .

Herbicide treatments and HPLC analysis

For herbicide-containing agar plates, autoclaved MS agar was cooled to 40°C , stock solutions of the corresponding chemicals were added and the media poured into Petri plates. The following substances were used: CPTA [2-(4-chlorophenylthio)triethylam-

monium chloride; synthesized according to Scheutz and Baldwin 1958]; norflurazon (SAN 9789, 4-chloro-5-(methylamino)-2-(α,α -trifluoro-m-tolyl)-3(2H)-pyridazinone; Mayer et al. 1989), gabaacilic (2-amino-2,3-dihydrobenzoxazole acid; Sigma).

Seeds from *A. thaliana* (ecotype Wassilewskaja) were surface sterilized and plated on MS agar plates and herbicide-containing MS agar plates. After vernalization for 4 days at 4°C , seeds were germinated for 3 days in darkness and thereafter illuminated for 24 h with W light. Seedlings were harvested, immediately frozen in liquid nitrogen, ground to powder and resuspended in 500 μl 100 mM Tris. An aliquot of 50 μl was taken for protein determination (Bradford 1976). The remaining suspension was extracted with an equal amount of CHCl_3 /methanol (2/1, v/v). The organic phase was separated by centrifugation (5 min, 10,000 g), collected and the aqueous phase was re-extracted twice with CHCl_3 . The collected organic phases were subjected to quantitative HPLC analysis. As internal standards, lycopin (Hoffmann-La Roche, Basel, Switzerland) was used for control seedling and seedlings treated with norflurazon and gabaacilic, whereas β -carotene (Sigma, Germany) was used for CPTA-treated seedlings. The HPLC-system consisting of a C30 reverse phase column (YMC 200, CROM, Herrenberg, Germany) and a gradient system using (A) methanol/*tert*-butyl-methyl ether/ H_2O (75/15/15, by volume) as the polar solvent and (B) methanol/*tert*-butyl-methyl ether (50/50, v/v) as the nonpolar solvent. The gradient profile was 100% A linear to 0% A in 100 min, then isocratic for an additional 10 min at a constant flow-rate of 1 ml/min. UV/VIS spectra were monitored by a photodiode array detector (Waters 986, Eschborn, Germany). For analysis the Millennium software package version 2.1 (Waters) was used. Products were identified by chromatographic comparison to authentic reference substances isolated from *tangerine* tomatoes (Clough and Pattenden 1979) and by their spectral characteristics.

Results

Cloning of the *psy* gene and spatial pattern of expression of the luc-reporter gene

The *psy* gene was isolated by screening a genomic library of *Arabidopsis thaliana* using a fragment of the *A. thaliana psy* cDNA (Bartley and Scolnik 1994) as a probe. This yielded a 7-kb DNA fragment which revealed by sequencing the presence of the *psy* gene as part of chromosome 5 (GenBank accession number AB005238 from bp 18150 to bp 25521, Sato et al. 1997). The fragment contained the complete transcribed region of 3.1 kb, 525 bp of the 3'-untranscribed region and 3.7 kb of the *psy* promoter region. Figure 1 shows the DNA sequence of the *psy* promoter region up to position -1746. Position +1 represents the first nucleotide of the longest cDNA isolated. Two putative TATA motifs are localized at positions -124 and -153.

To investigate regulatory regions within the promoter, we constructed six different translational fusions using the luciferase gene as the reporter at the following sites: -1746, -1314, -910, -809, -300 and -196. The fusions -1746/+716, -1314/+716, -300/+716 and -196/+716 were cloned into the vector pBIN121 upstream of the *nos* polyadenylation signal. These constructs were used for *Agrobacterium*-mediated transformation of *A. thaliana*. Homozygous inbreds were produced from several lines of all transformants to be used for expression analysis.

Fig. 1 Sequence of the *psy* promoter and 5' non-translated region. Position +1 represents the first nucleotide of the longest cDNA isolated (Bartley and Scolnik 1994). Exons are shown in uppercase letters and the first intron is italicized. Breakpoints of the fusions with the *luc* reporter gene are indicated by arrows and underlined letters. Putative TATA boxes are shown in bold, the ATG start codon is underlined. The region represents the sequence from 21076 to 25521 of the P1 clone MKP11 of chromosome 5 (GenBank accession number AB005238)

→1746		
gtagaattagggcgggttgacataaatttggtag	aaacacatactttctacatcgtacataacacac	-1876
aaagcgggtttctttacggagaaatgttatat	cagatagcggagaaatacggagacagcgtacac	-1406
accagaggaataaaccacacacagagaaactta	aaagacacacacacacacacacacacacacac	-1536
aaagtgtgcagagcagctatgggacacacacacac	taagagagacacacacacacacacacacacac	-1466
tgaaacttgacacacacacacacacacacacac	ctttacacacacacacacacacacacacacac	-1396
tatcgctctcctcctggcgatgggtacacacacac	ctctctctctctctctctctctctctctctctct	-1326
→1314		
cccaaatggcgtgacacacacacacacacacac	tgctctctctctctctctctctctctctctctct	-1256
tacatactctctctctctctctctctctctctct	tacatactctctctctctctctctctctctctct	-1186
actctctctctctctctctctctctctctctctct	tctctctctctctctctctctctctctctctct	-1116
aaagcgtctctctctctctctctctctctctctct	atagtgctctctctctctctctctctctctctct	-1046
gtagaaatttttagcagacacacacacacacac	tatactgtctctctctctctctctctctctctct	-976
→910		
acacatgatgtatctctctctctctctctctctct	atctctctctctctctctctctctctctctctct	-906
tgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt	tttttttttttttttttttttttttttttttttt	-836
→809		
taaatctacacacacacacacacacacacacac	ctctctctctctctctctctctctctctctctct	-746
aatctatctctctctctctctctctctctctctct	ctctctctctctctctctctctctctctctctct	-696
acgatttgatgacacacacacacacacacacac	tttttttttttttttttttttttttttttttttt	-626
atgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt	aaagtttagacacacacacacacacacacacac	-556
tacacacacacacacacacacacacacacacac	aaacacacacacacacacacacacacacacac	-486
tctctctctctctctctctctctctctctctctct	aaagacacacacacacacacacacacacacac	-416
atctctctctctctctctctctctctctctctctct	gaataacacacacacacacacacacacacac	-346
→300		
atgtatgagacacacacacacacacacacacac	ataatgtgtgtgtgtgtgtgtgtgtgtgtgtgt	-276
tacatgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt	ctctctctctctctctctctctctctctctctct	-206
→196		
cagcagctctctctctctctctctctctctctct	aaacacacacacacacacacacacacacacac	-136
aaacacacacacacacacacacacacacacac	ttctctctctctctctctctctctctctctctct	-66
→11		
caagagagagagagagagagagagagagagag	ttctctctctctctctctctctctctctctctct	4
tggtctctctctctctctctctctctctctctct	aaacacacacacacacacacacacacacacac	14
gtcaaacctctctctctctctctctctctctctct	ttctctctctctctctctctctctctctctctct	24
caaaagtctctctctctctctctctctctctctct	gagaaagagagagagagagagagagagagag	214
cyctctctctctctctctctctctctctctctct	cactgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt	284
tggtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt	ttctctctctctctctctctctctctctctctct	354
tgagcagcagcagcagcagcagcagcagcagcag	ctctctctctctctctctctctctctctctctct	424
tgctctctctctctctctctctctctctctctct	tgctctctctctctctctctctctctctctctct	494
tgctctctctctctctctctctctctctctctct	ctctctctctctctctctctctctctctctctct	564
agcgtctctctctctctctctctctctctctctct	ttctctctctctctctctctctctctctctctct	634
taactctctctctctctctctctctctctctctct	ttctctctctctctctctctctctctctctctct	704
→716		
aaactctctctctctctctctctctctctctctct		716

In plants containing the -1746/+716 *psy/luc* transgene, luciferase activity was detected in all tissues tested, including those which normally contain carotenoids only in trace amounts if any, such as roots (Fig. 2). The highest luciferase activity was measured in flowering buds and ripening seed pods while intermediate values were obtained for leaves.

Expression during photomorphogenesis

As we have shown previously, the phytochrome system mediates the light induction of PSY as observed at the transcript as well as at the protein level (von Lintig et al. 1997; Welsch et al. 2000). Using the different transgenic *psy/luc Arabidopsis* lines, we aimed at characterizing *cis*-acting elements within the *psy* promoter responsible for light induction. For this purpose, luciferase activity was determined from seedlings which were etiolated for 3 days and from etiolated seedlings illuminated for 24 h with different light qualities (Fig. 3). The longest promoter fragment used (-1746/+716) showed two- to threefold increases in luciferase activity after illumination with W, FR, R and B light. This correlates well with the increase of *psy* transcript amounts in *Arabidopsis* seedlings subjected to the same light treatment (von Lintig et al. 1997).

Transformants carrying the -1314/+716 *psy/luc* fusion or the -1746/+716 *psy/luc* fusion were almost indistinguishable in their patterns of luciferase activity. However, further truncation up to position -300 led to a decrease of 20–30% in promoter activity under all light conditions. Further truncation up to -196 abolished the induction under R light completely, whereas an induction under W, FR and B light was still observed. This indicates the existence of spatially separated *cis*-acting elements responsible for R and FR/B responses: *cis*-acting elements located between -300 and -196 are essential for responses to R light while those for FR and B light response are located in the proximity of the TATA box, up to position -196. Furthermore, since both the -300/+716 and -196/+716 *psy/luc* fusions show the same luciferase activity in etiolated seedlings, the responsible *cis*-acting elements are also localized in the proximity of the TATA box region, up to -196.

Thus, two regions within the *psy* promoter appear to be mainly involved in the regulation, one being located between -1314 and -300 (heretofore TATA box distal region) responsible for a basal strong promoter activity, the other being located between -300 and the TATA box (heretofore TATA box proximal region). This latter region is mainly involved in the differentiated response towards different light qualities.

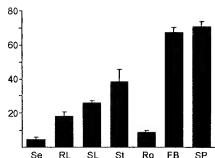


Fig. 2 Activity of the -1746/+716-*psyluc* fusion in tissue extracts from 6-week-old transgenic *Arabidopsis* plants. Luciferase activity was determined by a luminometric assay and is expressed in relative light units (RLU) s⁻¹ μg⁻¹ protein (FB flowering buds, RL rosette leaves, Ro roots, Se seeds, SL stem leaves, SP seed pods, St stem)

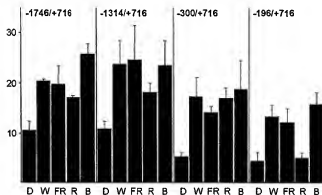


Fig. 3 Activity of different *psyluc* fusions in transgenic *Arabidopsis* seedlings during de-etiolation. The numbers indicate the breakpoints of the *psyluc* promoter region fused with the *luc* gene. Transgenic *Arabidopsis* seedlings were etiolated for 3 days (D) and thereafter illuminated for 24 h with white (W), far-red (FR), red (R) and blue (B) light. Luciferase activity was measured luminometrically and expressed in RLU s⁻¹ μg⁻¹ protein

Effects of herbicides on the expression

Herbicides that interfere with carotenoid formation have been considered to affect the regulation of carotenoid biosynthesis in a sort of feedback mechanism (Corona et al. 1996; Al-Babili et al. 1999). Therefore, the influence of different herbicides on carotenoid content and on luciferase activity in herbicide treated *psyluc* plants was analyzed (Fig. 4). To include possible effects of herbicides on light induction, the herbicide treatment was carried out with etiolated seedlings as well as with seedlings illuminated for 24 h with W light. The two compounds acting on carotenogenic enzymes were norflurazon, which inhibits PDS and leads to phytoene-accumulation, and CPTA, which inhibits lycopene cyclase and yields lycopene. Furthermore, to compare the effects of gabaculine on the *pds* promoter reported in the literature (Corona et al. 1996), we included this compound into our studies. Gabaculine is an inhibitor of chlorophyll biosynthesis acting at the level of the

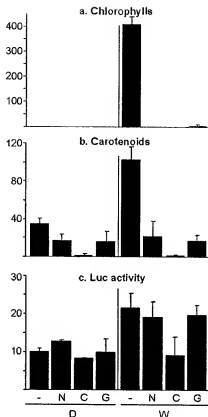


Fig. 4a-c Effects of herbicide treatments on carotenoid and chlorophyll content and *psyluc* promoter activity in *Arabidopsis* seedlings. Wt and transgenic -1746/+716 *psyluc Arabidopsis* seedlings were etiolated for 3 days (D) and thereafter illuminated for 24 h with white (W) light. Chlorophyll (a), carotenoid content (b) (ng μg⁻¹ protein) and luciferase activity (RLU s⁻¹ μg⁻¹ protein) are shown. Data are means ± SE of two experiments except for c where data are means of six experiments (C CPTA, G gabaculine, N norflurazon)

enzyme glutamate 1-semialdehyde aminotransferase (Werck-Reichhart et al. 1988).

As determined by quantitative HPLC analysis, in etiolated seedlings treated with norflurazon and gabaculine, the carotenoid content reached only half of the amount present in the untreated controls (Fig. 4b). Furthermore, the carotenoid content remained constant after illumination in herbicide-treated seedlings, whereas a tripling of carotenoids occurred in untreated seedlings. In seedlings grown on CPTA, the carotenoid amount was further decreased and consisted of only traces of lycopene. As with all other herbicides tested, this remained unchanged after illumination. Thus, in this system, all herbicides used led to a decrease in carotenoid content and a complete loss of light-induced accumulation.

This loss, however, is not related in all cases to equivalent responses in promoter activity (Fig. 4c). Interestingly, the decrease in the carotenoid content affected by norflurazon and gabaculine in the dark did

not significantly change the luciferase activity in -1746/+716 *psy/luc* plants. This indicates that, contrary to that observed for the tomato *pds* promoter (Corona et al. 1996), there was no regulatory feedback mechanism acting in the presence of these two compounds. However, the regulation of signal transduction pathways leading to light induction of the *psy* promoter occurred undisturbed, since an increase in luciferase activity was observed in the respective illuminated seedlings.

CPTA treatment did not change reporter activity in dark-grown seedlings but, in contrast to norflurazon and gabaclucine treatment, it abolished light induction almost absolutely.

Characterization of *cis*-acting elements of the TATA box proximal promoter region

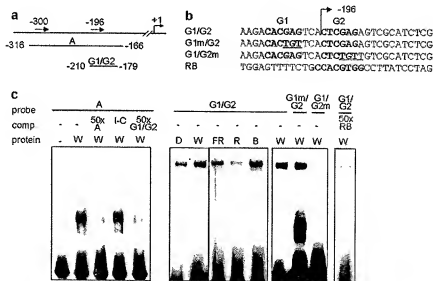
The analysis of luciferase expression of the *psy/luc* fusions led to the conclusion that *cis*-acting elements mediating differential light responses are located within the TATA box proximal promoter region up to position -300. To further characterize these elements, EMSAs were performed using nuclear extracts from *Sinapis alba* seedlings. Using the region from -316 to -166 as a probe, the formation of specific protein/DNA complexes was observed, as shown by successful competition of complex formation with the same unlabelled probe (Fig. 5c, left). As the competition occurred also with a shorter promoter fragment corresponding to position -210 to -179, it was concluded that the corresponding *cis*-acting elements are located within this region. This finding was confirmed by EMSAs using this promoter sequence as a probe (Fig. 5c, middle). Furthermore, the formation of protein/DNA complexes correlated with the promoter activities observed under different light conditions. Extracts from seedlings illuminated with W, FR and B light showed higher binding activity than nuclear extracts from etiolated seedlings. However,

when obtained from R-light-illuminated seedlings, only weak protein/DNA complexes were formed.

Although motifs of well-known plant transcription factors were not found within this -210 to -179 promoter region, two short motifs share some similarity to G-box motifs, which are known to be involved in the light regulation of several genes (in the following G1 and G2, see Fig. 5b; Giuliano et al. 1988). According to Schindler et al. (1992), mutations in these motifs affect the affinity towards GBFs. Therefore, we investigated possible functions of these two motifs by analyzing the binding activities of DNA fragments successively mutated in these motifs (Fig. 5c, right). The mutation in motif G1 led to the appearance of an additional protein/DNA complex with different migration behavior, whereas the mutation in G2 prevented complex formation completely. Thus, both motifs are necessary for the formation of the protein/DNA complex, but to a different extent: while G2 is essential for the formation, G1 contributes more to complex stability. This may be reflected in the fact that the -196/+716 *psy/luc* fusion, which includes G2, but not G1, shows slightly lower light induction than the -300/+716 *psy/luc* fusion and has lost R induction.

The fact that mutations in both motifs effected complex formation indicated the involvement of GBFs. A further clue was obtained by a competition assay us-

Fig. 5 a Fragments from the TATA box proximal region of the *psy* promoter used in electrophoretic mobility shift assays (EMSA). The numbers depict the distance in bp to the transcription start site (+1). b Sequences of the oligonucleotides used for the EMSAs. G-box-like elements and the G-box of the *rbcs3B* promoter are shown in bold face; mutations are given as underlined. G1/G2 is the *psy* promoter region between -210 and -179; RB is the *rbcs3B* promoter region between 8212 and 8242 (Dedonder et al. 1993). c EMSAs with mustard nuclear extracts from etiolated (D) and far-red (FR), red (R), blue (B) and white (W) light illuminated mustard seedlings. Competitions were performed with the molar excess of unlabelled fragments as given [*I*-C 250 ng poly(dIdC) was added to the incubation]



sequence ATCTA seemed indicative (Fig. 7b). Therefore, we investigated the possible involvement of the resulting sequence motifs on complex formation. Both motifs were successively mutated and these fragments were used as competitors. As shown in Fig. 7c (middle), individual mutation of the first and of the second ATCTA motif both led to a decrease in binding activity, while simultaneous mutations in both motifs completely abolished binding ability. Therefore, the sequence ATCTA represents at least in part the *cis*-acting element for a transcription factor mediating a strong *psy* promoter activity.

To examine the distribution of this motif, we screened the promoter regions of genes from different organisms expected to be expressed coordinately with carotenoid biosynthesis during the formation of photosynthetic complexes (see Table 1). Interestingly, the ATCTA motif was found not only in promoters driving the expression of proteins involved in the carotenoid pathway, like deoxyxylulose-phosphate synthase and PDS, but it occurred also in genes in the tocopherol biosynthesis pathway, such as for hydroxyphenyl-pyruvate dioxygenase, geranylgeranyl-diphosphate reductase, prenyl transferase and γ -tocopherol methyltransferase. Furthermore, it was found in promoters of genes involved in photosynthesis, such as the ones coding for the chlorophyll a/b binding protein (CAB) and for plastocyanin (PC).

In order to investigate whether these ATCTA-containing promoter regions also lead to complex formation, EMSAs were conducted using nuclear extracts from mustard seedlings illuminated with W light. Promoter

regions from the following genes were used as radiolabelled probes: *pds* from *Lycopersicon esculentum* (GenBank accession number U46919), *cab* from *S. alba* (GenBank accession number X16436) and *A. thaliana* (GenBank accession number J04098) and *pc* from *Pisum sativum* (GenBank accession number X16082). Consistent with the competition assays using promoter fragments mutated in one of the two ATCTA elements, these studies revealed that the tandem arrangement of the ATCTA motif was not necessary to allow complex formation. Among the promoters investigated, such an arrangement is observed only in the *cab/S. alba* and in the *pc/P. sativum* promoters, while occurring only singly in the others (see also Table 1). Fig. 8 shows the formation of protein/DNA complexes for all promoter regions tested. Their electrophoretic mobility corresponded to that observed with the probe from the *psy* promoter but differing somewhat in the amounts of retarded radiolabelled probe. The complex formed with the ATCTA motif-containing region from the *cab* promoter of *S. alba* can be competed by the corresponding ATCTA motif-containing region from the *psy* promoter, suggesting the involvement of the same transcription factor.

Discussion

The formation of a functional photosynthetic apparatus requires the synthesis of carotenoids to be well

Table 1 The ATCTA element in different promoters of plastid-localized proteins. The position of the ATCTA element is denoted relative to translational (Tt) or transcriptional start (Tx) of the gene. Numbers in bold indicate ATCTA elements of sequences which were used as radiolabelled probes for EMSAs in Fig. 8. GenBank accession numbers for the promoter sequences analyzed are as follows: DXS, A1161542; PSY, A060238; PDS (*Lycopersicon esculentum*), U46919; PDS (*Zea mays*), AF039585; CAB

(*Sinapis alba*), X16436; CAB1 (*Arabidopsis thaliana*), J04098; PC (*Pisum sativum*), X16082; PC (*Arabidopsis thaliana*), S67901; PC (*Hordeum vulgare*), Z28347; HPD, NC_003070; GGR, AC011765; PT, AC007651; TMT, AC006193 (CAB chlorophyll a/b-binding protein, DXS deoxyxylulose-phosphate synthase, GGR geranylgeranyl-diphosphate reductase, HPD hydroxyphenyl-pyruvate dioxygenase, PC plastocyanin, PDS phytyl desaturase, PT prenyl transferase, TMT γ -tocopherol methyltransferase)

Proteins	Gene	ATCTA element	Sequence
Carotenoid biosynthesis	<i>dxs</i> (<i>A. thaliana</i>)	-819Tt -418Tt	ATTITTTCTGTAAACATCTAAAAATTTAT AAATAATATCATCAATATCTATCCAAAAC TTAATCAACTCAAAATATCTAAATATAAT TGTTTGGAGTTTATTTATCTAAAGTAAAC AATAAACATCTAATTAATCTAAAAAGCAAT CTATATACTGTCTATATCTATATTTAAT
	<i>psy</i> (<i>A. thaliana</i>)	-841Tx	TAATCTAAATCTGAAATATCTAAATGTGTTA TACTCTAAATCTACGGATCTAATCTCGAG AATGTGTTTAAGTATAGATATCTATCGTCTCA TAAATTTATAGTTTATCTACTTTGTTC
	<i>pds</i> (<i>L. esculentum</i>)	-871Tx	TGAACGACCACTAGATATCTAAACACAT GTGGACATCTACATATCTAAATCTACAT TTTTATAAGATAATGTATCTAGGTTTGCT
	<i>pds</i> (<i>Z. mays</i>)	-327Tx -185Tx	TGTTCAAAAGTCTCTATCTCATCTATGCA AATAACTGCAATTTTTATCTAAACCAATA ATACATGTAGACCAAAATCTAAAGGTGTTT TCATGGCACATAGAAATATCAAGAACTGT
	<i>cab</i> (<i>S. alba</i>)	-1159Tt -1132Tt	TTCTCTTACTAAAAATATCTAAAAATCAT GGAATCTCTCAACAAATATCTAATCCACTA TATGAAACCAATTTAAATCTAGAAATTTTC AGTACCATCTCAAGATATCTAAAAAATGT GAGTGAATGATATTTATCTAAACAAATG
Photosynthesis	<i>cab1</i> (<i>A. thaliana</i>)	-490Tt -248Tt	
	<i>pc</i> (<i>P. sativum</i>)	-101Tx	
	<i>pc</i> (<i>A. thaliana</i>)	-1159Tx -149Tx	
	<i>pc</i> (<i>H. vulgare</i>)	-499Tx	
	<i>hpd</i> (<i>A. thaliana</i>)	-894Tt -400Tt	
Tocopherol biosynthesis	<i>ggr</i> (<i>A. thaliana</i>)	-1001Tt -124Tt	
	<i>pt</i> (<i>A. thaliana</i>)	-908Tt -151Tt	
	<i>tmt</i> (<i>A. thaliana</i>)	-111Tt	

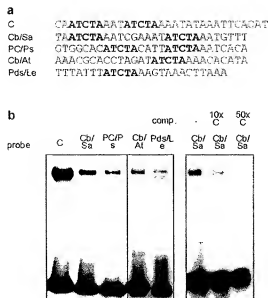


Fig. 8 a Sequences of the oligonucleotides used in the EMSAs. ATCTA elements shown in bold (C *psy* promoter region between -856 and -825, Cb/Sa *cab* promoter/Sinapis alba, PC/Ps plastocyanin promoter/Pisum sativum, Cb/At *cab1* promoter/A. thaliana, Pds/Le *psd* promoter/Lycopersicon esculentum). For GenBank accession numbers and positions within the promoters, see Table 1. b EMSAs with labeled oligonucleotides indicated in A and nuclear extracts from mustard seedlings illuminated with white light. Competitions were performed with 10x and 50x molar excess of unlabeled fragment C

coordinated with all other constituents involved in photosynthesis. The enzyme phytyl synthase (PSY) catalyzes the first reaction specifically devoted to carotenoid formation. Therefore, the finding that PSY represents the first light-induced step in the pathway on the transcriptional, protein and enzymatic level appears indicative (von Lintig et al. 1997; Welsch et al. 2000). As a consequence, we decided to focus on the *psy* promoter from *Arabidopsis thaliana* and analyzed transgenic plants carrying promoter/luciferase fusions.

The activity of the *psy* promoter observed in the dark correlates well with the PSY accumulation in the prolamellar bodies of etioplasts. Illumination with W light for 24 h induces a doubling of PSY protein (Welsch et al. 2000); concomitantly *psy* promoter activity doubles, as we show here (Fig. 3). This parallelism in transcript and protein levels indicates that translation is not involved in controlling *psy* expression. However, post-translational mechanisms are involved: it must be kept in mind that the increased promoter activity observed under FR light, for instance, yields an enzymatically inactive protein requiring the decay of the prolamellar body and the development of thylakoid membranes for activity. In one interpretation, both *psy* promoter activity in the dark and enzymatic activation by light-induced formation of membrane structures represent a synergistic mechanism to guarantee a rapid supply of photoprotective carotenoids during the initial phase of photomorphogenesis.

Nonetheless, with the results presented it can be stated that regulation of carotenoid biosynthesis is subjected to strong transcriptional regulation. This supports several studies of the steady-state concentrations of carotenogenic mRNAs during developmental processes in different systems (reviewed in Hirschberg et al. 1997).

In adult plants, the pattern of tissue-specific promoter activity did not meet expectations in all cases (Fig. 2). For example, the signal obtained for roots is surprising but may be related to a basal level of carotenoid biosynthesis. Carotenoids produced here may represent abscisic acid (ABA) precursors subjected to dioxygenase-catalyzed cleavage (Schwartz et al. 1997); in fact, ABA synthesis occurs also in roots (Milborrow 2001). The high level of *psy* promoter activity observed in developing seed pods may also be linked with ABA formation, as this phytohormone is known to prevent premature germination.

Inflorescences showed a high expression of the *psy/luc* transgene. But, while separate analysis of flower tissues is difficult in *Arabidopsis*, there are reports from tomato and tobacco expressing *gus* under control of the *pds* promoter. Here, anthers showed the strongest expression of the *pds/gus* transgene (Corona et al. 1996), followed by corollae and pistils. High expression in anthers was related to a structural function of carotenoids discussed previously (Brooks and Shaw 1968).

Carotenoid biosynthesis takes place exclusively in plastids, catalyzed by enzymes that are encoded by nuclear genes. It is well known that inhibition of plastid development may affect the expression of such proteins. For instance, plastid defects induced by norflurazon lead to differential alterations in the expression of the nuclear-encoded plastid proteins in barley seedlings (Batschauer et al. 1986). Therefore, we studied the effects of norflurazon, CPTA and gabaculine on the expression of the reporter gene. Norflurazon, a classical bleaching herbicide, and gabaculine, an inhibitor of chlorophyll biosynthesis, did not affect *psy* promoter activity during etiolation or after illumination (Fig. 4). This is quite surprising, at least in the case of norflurazon, since increased activity would be expected as a result of the photo-oxidative damage it exerts. Thus, neither the accumulation of phytoene precursors nor the reduction of final carotenoid products seems to exert notable feedback regulatory activity. In this respect, regulation of the *psy* promoter and the *pds* promoter from tomato differ; for the latter, an increase in promoter activity was reported in response to these two compounds (Corona et al. 1996). These results agree with the absence of significant effects of norflurazon treatment on the steady-state levels of the tomato *psy* mRNA (Giuliano et al. 1993). Similarly, the lycopene cyclase inhibitor CPTA, leading to lycopene accumulation, was reported to mediate a positive *pds* promoter response in the dark and in the light, whereas in our experiments the *psy* promoter did not respond to CPTA in the dark. Interestingly, CPTA treatment completely abolished the ability of this

promoter to respond positively to light. It remains to be clarified whether this is due to feedback-signaling initiated by the accumulated lycopene. Such a role for lycopene can be concluded from the unexpected results obtained with rice endosperm expressing carotenoid biosynthetic enzymes. Here, formation of *trans*-lycopene, mediated by *psy* and a bacterial *crtI* (a *trans*-lycopene forming carotene desaturase) led to the establishment of the entire carotenogenic pathway including the formation of xanthophylls (Ye et al. 1999). Similarly, expression of *crtI* in tomatoes did not lead to increased synthesis of lycopene, but to an increase in β -carotene. Endogenous carotenoid genes were concurrently up-regulated, except for *psy*, which was repressed (Romer et al. 2000). This downregulation of *psy* correlates with the effect of *trans*-lycopene on the *psy* promoter demonstrated here but conflicts with the results of CPTA experiments with daffodil flowers, where lycopene accumulation increased the abundance of *psy* transcripts and protein (Al-Babili et al. 1999).

Light induces the accumulation of carotenoids. At the level of the *psy* promoter, all light qualities tested in the present investigation increased *psy* promoter-mediated luciferase activity in transgenic seedlings carrying the -1746/+716 *psy/luc* fusion (Fig. 3). The strongest induction was observed for B light, which indicates a possible important contribution by cryptochromes. The phytochrome system is also involved: analysis of *phyA* and *phyB* mutants of *Arabidopsis* demonstrated earlier that PHYA is involved in the FR induction of *psy* transcript amounts (von Lintig et al. 1997). In agreement with these findings, FR light led to a strong increase in promoter activity. A similar response pattern toward different light qualities, albeit to a lower extent than the -1746/+716 *psy/luc* fusion, is mediated by the -300/+716 *psy/luc* fusion. However, truncation of 104 bp leading to the -196/+716 *psy/luc* fusion abolished the induction in R light completely. This indicates that *cis*-acting elements involved in the phytochrome response under R light are located between -300 and -196 of the promoter, whereas elements mediating responses to FR and B light as well as a residual promoter activity in the dark are located in the first 196 bp. Within the -300 to -196 promoter region, the position of two spatially separable *cis*-acting elements could be restricted to between positions -210 and -179 (see Fig. 5). The amounts of protein/DNA complexes formed with this region using nuclear extracts from etiolated seedlings and from FR, B and W light illuminated seedlings corresponded to the *psy* promoter activity measured under these conditions. Therefore, the binding activities of the *trans*-acting factors involved here are regulated by FR- and B-light-receptor mediated mechanisms.

The induction under R light seems to involve different *cis*-acting elements. Under R light, only weak complex formation was detectable using the -210/-179 promoter sequence as a probe. Consistent with this finding, the -196/+716 *psy/luc* fusion, which disjoins the two *cis*-acting elements characterized, lost the R induc-

tion. Therefore, additional *cis*-acting elements downstream of position -179 are required to explain the observed strong R induction in the -300/+716 *psy/luc* fusion.

The most intensely investigated group of transcription factors involved in light regulation are the GBFs (Giuliano et al. 1988; Menkens et al. 1995). A competition assay with the perfect palindromic G-box of the *rbcS3b* promoter of *A. thaliana* demonstrated the involvement of GBFs in the protein/DNA complexes formed within this *psy* promoter region (Fig. 5). Known mechanisms involved in increased GBF binding activity, such as light-induced translocation from the cytoplasm (Harter et al. 1994) or phosphorylation (Klimczak et al. 1992, 1995), may be responsible for the effects observed.

The *psy* promoter region from -215 to -166 contains two G-box-like motifs (G1 and G2). As shown by EMSAs with probes carrying mutations in G1 and G2, both motifs are involved in the formation of the protein/DNA complex observed (Fig. 5). Since GBFs belong to the group of basic leucine zipper transcription factors, it is generally assumed that a central ACGT core is necessary for binding (Foster et al. 1994) while flanking sequences determine sequence specificity (Williams et al. 1992; Izawa et al. 1993). The G-box-like motifs in the *psy* promoter do not meet this criteria, because they contain the sequences ACGA and TCGA in G1 and G2, respectively. However, there are some indications that a central ACGT core is not obligatory (de Pater et al. 1994; Yunes et al. 1994). The exact identity of the GBFs involved in the binding to *psy* promoter G1 and G2 remains to be investigated.

The light-dependent activity mediated by the -300/+716 promoter region is not qualitatively but quantitatively modulated by *cis*-acting elements located further upstream, as reflected by the strong luciferase activity in the -1314/+716 *psy/luc* plants. Quantification of transient luciferase expression revealed these elements to be located between positions -910 and -809 (Fig. 6). Competition EMSAs with nuclear extracts from mustard seedlings illuminated with W light restricts localization of the corresponding *cis*-acting elements to a region between -856 and -825. The amounts of protein/DNA complexes formed with this promoter region were not affected markedly by nuclear extracts from differently illuminated mustard seedlings (Fig. 7). Thus, the corresponding *trans*-acting factors are constitutively required for a high basal level of *psy* promoter activity.

The sequence of the DNA fragments used in these EMSAs contained two ATCTA motifs in tandem. Competition EMSAs with DNA fragments carrying the mutated motifs demonstrated that these boxes indeed bind a *trans*-acting factor (Fig. 7) and that the existence of only one ATCTA motif is sufficient. Mutation of only one motif was insufficient to prevent complex formation, while mutation of both led to a complete loss of binding capacity.

Apart from in the *psy* promoter, the ATCTA motif also occurs in promoters of genes involved in carotenoid

and tocopherol biosynthesis, and also in some photosynthesis-related genes (see Table 1). This may indicate a more general importance of this motif in the co-regulation of these genes. This is further corroborated by the observation that different ATCTA-containing promoter regions in EMSAs resulted in protein/DNA complexes with identical migration behavior but in different amounts (Fig. 8). The ATCTA element characterized here shares some similarity to a cis-acting element found in several *cab* genes which are recognized by CCA1. This myb-related transcription factor was shown to be involved in the phytochrome induction of these genes (Wang et al. 1997; Wang and Tobin 1998). However, footprint analysis defined the sequence AA⁴/CAATCT as the binding sequence for CCA1, whereas the presence of A residues upstream of the ATCTA element is not necessary for binding of the transcription factor involved here. This can be concluded both from the competition assay using mutated ATCTA sequences (Fig. 7c), as well as from the sequences neighboring the ATCTA elements found in other promoters (Fig. 8). Therefore, the involvement of an as yet unknown transcription factor seems probable.

Work is in progress towards the molecular identification of the corresponding transcription factor, to elucidate its potential for the synergistic regulation of different photosynthesis-related biochemical pathways.

Acknowledgements We thank the Arabidopsis Biological Resource Center, Ohio for the genomic library of *A. thaliana*. We thank P. Scolnik (DuPont, USA) for the *psu*-cDNA of *A. thaliana* and Dr. M. Rodriguez (group Prof. G. Neuhaus, Institute for Biology II, Albert-Ludwigs-University Freiburg) for providing pBIN35S-mGFP and pHG35SGUS. This work was supported by the Deutsche Forschungsgemeinschaft and in part by the European Community Biotech Programme (FAIR CT96, 1996–1999). R.W. was a member of the DFG Graduiertenkolleg (Molekulare Mechanismen pflanzlicher Differenzierung).

References

- Alba R, Cordonnier-Pratt MM, Pratt LH (2000) Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato. *Plant Physiol* 123:363–370
- Al-Babli S, Hartung W, Kleinig H, Beyer P (1999) CPTA modulates levels of carotenogenic proteins and their mRNAs and affects carotenoid and ABA content as well as chromoplast structure in *Narcissus pseudonarcissus* flowers. *Plant Biol* 1:607–612
- Bartley GE, Scolnik PA (1994) Nucleotide sequence of an *Arabidopsis* cDNA for phytoene synthase. *Plant Physiol* 104:1471–1472
- Batschauer A (1998) Photoreceptors of higher plants. *Planta* 206:479–492
- Batschauer A, Mosinger E, Kreuz K, Dorr I, Apel K (1986) The implication of a plastid-derived factor in the transcriptional control of nuclear genes encoding the light-harvesting chlorophyll *a/b* protein. *Eur J Biochem* 154:625–34
- Bechtold N, Ellis J, Pelletier G (1993) In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C R Acad Sci Paris* 316:1194–1199
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–54
- Brooks J, Shaw G (1968) Chemical structure of the xanthophyll of pollen walls and a new function for carotenoids in nature. *Nature* 219:532–533
- Camara B, Hugueney P, Bouvier F, Kuntz M, Monneret R (1995) Biochemistry and molecular biology of chromoplast development. *Int Rev Cytol* 163:175–247
- Clough J, Puttenden MG (1979) Naturally occurring poly-dicarotenoids. Stereochemistry of poly-dicarotenoids and its congeners in *tangerine* tomato fruits. *J Chem Soc Chem Commun* 616–619
- Corona V, Aracri B, Kosturkova G, Bartley GE, Pitto L, Giorgetti L, Scolnik P, Giuliano G (1996) Regulation of a carotenoid biosynthesis gene promoter during plant development. *Plant J* 9:505–512
- Dedonder A, Rethy R, Fredericq H, van Montagu M, Krebbers E (1993) *Arabidopsis rbcS* genes are differentially regulated by light. *Plant Physiol* 101:801–808
- Demmig-Adams B, Gilmore AM, Adams WW III (1996) In vivo functions of carotenoids in higher plants. *FASEB J* 10:403–412
- De Pater S, Katagiri F, Kijne J, Chua NH (1994) bZIP proteins bind to a palindromic sequence without an ACGT core located in a seed-specific element of the pea lectin promoter. *Plant J* 6:133–140
- Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11:1475–1489
- Feiner JJ, Vain P, Jones MW, McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Rep* 11:323–328
- Foster R, Izawa T, Chua NH (1994) Plant bZIP proteins gather at ACGT elements. *FASEB J* 8:192–200
- Frankhauser C, Chory J (1997) Light control of plant development. *Annu Rev Cell Dev Biol* 13:203–229
- Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. *Proc Natl Acad Sci USA* 85:7089–7093
- Giuliano G, Bartley GE, Scolnik PA (1993) Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* 5:379–387
- Harter K, Kircher S, Frohnmeyer H, Krenz M, Nagy F, Schäfer E (1994) Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. *Plant Cell* 6:545–559
- Henikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* 28:351–359
- Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. *Curr Opin Plant Biol* 4:210–218
- Hirschberg J, Cohen M, Harker M, Lotan T, Mann V, Pecker I (1997) Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. *Pure Appl Chem* 69:2145–2150
- Iida A, Nagasawa A, Oeda K (1995) Positive and negative cis-regulatory regions in the soybean glycinin promoter identified by quantitative transient gene expression. *Plant Cell Rep* 14:539–544
- Izawa T, Foster R, Chua NH (1993) Plant bZIP protein DNA binding specificity. *J Mol Biol* 233:1131–1144
- Jensen EO, Marcker KA, Schell J, de Bruijn F (1988) Interaction of a nucleotide specific, trans-acting factor with distinct DNA elements in the soybean leghaemoglobin *lbc* 5' upstream region. *EMBO J* 7:1265–1271
- Klenow H, Henningsen L (1970) Selective elimination of the exonuclease activity of the deoxyribonuclease acid polymerase from *Escherichia coli* by limited proteolysis. *Proc Natl Acad Sci USA* 65:168–175
- Klimczak LJ, Schindler U, Cashmore AR (1992) DNA binding activity of the *Arabidopsis* G-box binding factor GBFI is stimulated by phosphorylation by casein kinase II from brocoli. *Plant Cell* 4:87–98

- Klimczak LJ, Collinge MA, Farini D, Giuliano G, Walker JC, Cashmore AR (1995) Reconstitution of *Arabidopsis* casein kinase II from recombinant subunits and phosphorylation of transcription factor GBF1. *Plant Cell* 7:105-115
- Koncz C, Schell J (1986) The promoter of T₂-DNA gene 5 controls the tissue specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* 204:383-396
- Lin C (2000) Plant blue-light receptors. *Trends Plant Sci* 5:337-42
- Mayer PM, Bartlett DL, Beyer P, Kleinig H (1989) The in vitro mode of action of bleaching herbicides on the desaturation of 15-cis-phytoene and cis- ζ -carotene in isolated daffodil chromoplasts. *Pestic Biochem Physiol* 34:111-117
- Menkens AE, Schindler U, Cashmore AR (1995) The G-box: A ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends Biochem Sci* 20:506-510
- Milborrow BV (2001) The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. *J Exp Bot* 52:1145-1164
- Norris SR, Meyer SE, Callis J (1993) The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. *Plant Mol Biol* 21:895-906
- Rabbani S, Beyer P, von Lintig J, Huguency P, Kleinig H (1998) Induced β -carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant Physiol* 116:1239-1248
- Romer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM (2000) Elevation of the provitamin A content of transgenic tomato plants. *Nat Biotechnol* 18:666-669
- Sato S, Kotani H, Nakamura Y, Kaneko T, Asamizu E, Fukami M, Miyajima N, Tabata S (1997) Structural analysis of *Arabidopsis thaliana* chromosome 5. I. Sequence features of the 1.6-Mb regions covered by twenty physically assigned P1 clones. *DNA Res* 30:215-230
- Scheut RD, Baldwin RA (1958) The synthesis and properties of some substituted phenyl α -(N,N-dialkylamino)-alkyl sulfides. *Proc Acad Chem USA* 80:162-164
- Schindler U, Menkens AE, Bockmann H, Ecker JR, Cashmore AR (1992) Heterodimerization between light-regulated and ubiquitously expressed *Arabidopsis* GBF bZIP proteins. *EMBO J* 11:1261-1273
- Schulz B, Bennett MJ, Dilkes BP, Feldmann KA (1994) T-DNA tagging in *Arabidopsis thaliana*: cloning by gene disruption. *Plant Mol Biol Manual* K3. Kluwer, Dordrecht, pp 1-17
- Schwartz SH, Tan BC, Gage DA, Zeevaert JAD, McCarty DR (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* 276:1872-1874
- Van den Berg H, Faulks R, Fernando Granado H, Hirschberg J, Olmedilla B, Sandmann G, Southon S, Stahl W (2000) The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *J Sci Food Agric* 80:880-912
- Von Lintig J, Welsch R, Bonk M, Giuliano G, Batschauer A, Kleinig H (1997) Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *Plant J* 12:625-634
- Wang Z-Y, Tobin EM (1998) Constitutive expression of the Circadian Clock Associated 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93:1207-1217
- Wang Z-Y, Kenigsbuch D, Sun L, Harel E, Ong MS, Tobin EM (1997) An myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis thaliana* gene. *Plant Cell* 9:491-507
- Welsch R, Beyer P, Huguency P, Kleinig H, von Lintig J (2000) Regulation and activation of phytoene synthase, a key enzyme in carotenoid biosynthesis, during photomorphogenesis. *Planta* 211:846-854
- Werck-Reichardt D, Jones OT, Durst F (1988) Haem synthesis during cytochrome P-450 induction in higher plants. 5-Aminolaevulinic acid synthesis through a five-carbon pathway in *Helianthus tuberosus* tuber tissues aged in the dark. *Biochem J* 249:473-480
- Williams ME, Foster R, Chua NH (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. *Plant Cell* 4:485-496
- Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchey M (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. *Plant J* 19:249-57
- Yunes JA, Neto GC, da Silva MJ, Leite A, Ottoboni LMM, Arruda P (1994) The transcriptional activator Opaq2 recognizes two different target sequences in the 22-kD-like alpha-prolamin genes. *Plant Cell* 6:237-249

EXHIBIT B



Short communication

Identification of tomato *Lhc* promoter regions necessary for circadian expression

Birgit Piechulla*, Nicole Merforth and Barbara Rudolph¹

Universität Rostock, Biochemie, Fachbereich Biologie, Gertrudenstrasse 11a, 18051 Rostock, Germany (*author for correspondence); ¹present address: Institut für Pflanzenbau und Pflanzenzüchtung, Von-Siebold-Strasse 8, 37077 Göttingen, Germany

Received 21 April 1997; accepted in revised form 14 April 1998

Key words: circadian rhythm, light harvesting complex proteins, *Lhc* gene expression, promoter deletion analysis

Abstract

Expression of the light-harvesting complex protein genes (*Lhc*) is under the control of a circadian clock. To dissect the molecular regulatory components of the circadian clock a promoter deletion analysis of four tomato *Lhc* genes was performed in transgenic tobacco plants. The important 5'-upstream promoter regions are present at different positions relative to the transcription start site of *Lhc* b1*1, b1*2, *Lhc* a3 and *Lhc* a4. A short sequence of 47 nucleotides is necessary for conferring circadian *Lhc* mRNA oscillations. Sequence alignment of the specified promoter regions revealed a novel motif 'CAANNNNATC'. This motif is conserved in 5'-upstream regions of clock controlled *Lhc* genes and overlaps with a sequence relevant in phytochrome mediated gene expression.

Abbreviations: *Lhc* *alb*, genes encoding light-harvesting complex proteins (formerly *cab*, chlorophyll *a/b*-binding proteins); DD, continuous darkness; LD, light/dark conditions

Circadian rhythms have been described in nearly every eukaryotic organism as well as in some prokaryotes [19]. Despite the universal appearance and accumulated knowledge about the phenomenon of such rhythms, the molecular mechanism of the circadian clock is still not understood. Evidence is accumulating that indicates that the transmission of the biological clock occurs by feedback and autoregulation [28, 4]. To shed some light on the basic machinery we started to investigate possible components necessary for the circadian mRNA accumulation of the light-harvesting complex proteins (*Lhc*) of plants.

The light-harvesting complexes of plants are organized as protein-pigment units in the thylakoid membranes of chloroplasts which enhance the probability for light quantum absorption and focused channelling of the energy to the photosynthetic reaction centres [1, 16]. The proteins and pigments of these complexes and their respective genes have been intensively investigated in plants. Besides several important findings it

turned out that the synthesis of the proteins and the accumulation of respective mRNAs are under the control of a circadian clock [24, 27]. Interestingly, all 19 members of the tomato *Lhc* gene family exhibit this characteristic expression pattern [18]. Based on this similarity a common control mechanism is expected to function at the level of transcription of each tomato *Lhc* gene, for example *cis*- and *trans*-regulatory elements.

Several distinct *cis*-regulatory motifs have been detected for plant genes [22, 29]. A prominent element is the 'ACGT', the G-box, first described by Giuliano *et al.* [13], which is present in the promoter of plant genes encoding very different proteins [31]. However, the *trans*-acting factors binding to this motif belong to the same bZip type [3, 11, 15]. Another sequence ('CCTATCAT') has been described and correlated with light-responsive expression of several plant genes [14] as well as the 'GATA' motif (l-box). The latter, described by Castresana *et al.* [8], is present

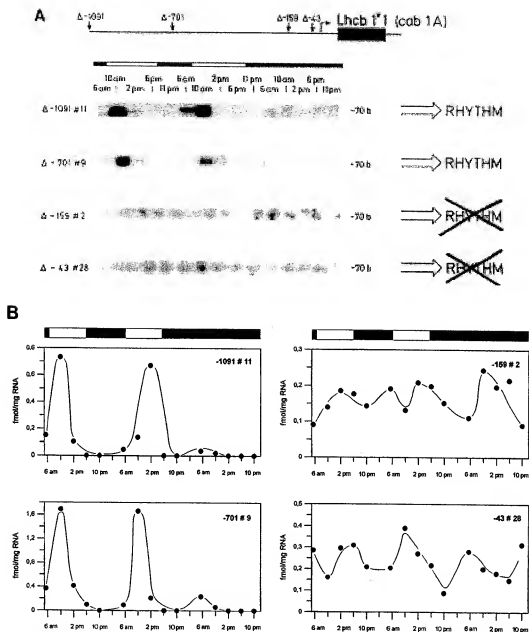


Figure 1. Steady-state mRNA levels of the tomato *Lhc b1*1* (*cab 1A*) gene in transgenic tobacco. **A.** Transgenic tobacco plants carrying the deleted tomato *Lhc* gene promoter ($\Delta-1091$, $\Delta-701$, $\Delta-159$, $\Delta-43$) were grown in light/dark (light: 06:00–18:00) and continuous dark conditions. Leaves were harvested at indicated time points and tomato *Lhc* mRNAs were detected with the primer extension technique. The length of the extended primer is 70 bp. **B.** mRNA levels of deletion constructs were quantitated (fmol per mg RNA) and data of representative tobacco lines are presented.

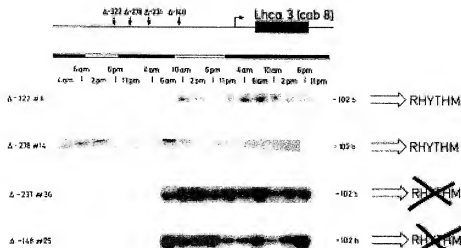


Figure 2. Steady-state mRNA levels of the tomato *Lhc a3* (*cab 8*) gene in transgenic tobacco. Transgenic tobacco plants carrying the deleted tomato *Lhc* gene promoter (Δ -322, Δ -278, Δ -231, Δ -148) were grown in light/dark (light: 06:00–18:00) and continuous dark conditions. Leaves were harvested at indicated time points and tomato *Lhc* mRNAs were detected with the primer extension technique. The length of the extended primer is 102 bp.

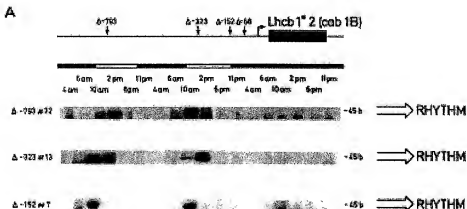


Figure 3. Steady-state mRNA levels of the tomato *Lhc b1*2* (*cab 1B*) gene in transgenic tobacco. A. Transgenic tobacco plants carrying the deleted tomato *Lhc* gene promoter (Δ -793, Δ -323, Δ -152) were grown in light/dark (light: 06:00–18:00) and continuous dark conditions. Leaves were harvested at indicated time points and tomato *Lhc* mRNAs were detected with the primer extension technique. The length of the extended primer is 45 bp. B. mRNA levels of the deletion constructs were quantitated (fmol per mg RNA) and data of representative tobacco lines are presented.

in the promoter of several light-harvesting complex proteins (LHCP) [12, 25], of the small subunit of Rubisco [9] and in the CaMV 35S promoter [21]. In the case of many *Lhc* gene promoters this motif is two to four times repeated. The nucleotide distances between the 'GATA' sequences are highly conserved [25]. Since the discovery of this motif and its abundant appearance a role as a regulatory unit had been

postulated. Variation of the nucleotide sequence of this motif indicated that the 'GATA' element modulates the transcription positively rather than negatively [12]. A correlation of light- and/or tissue-specific expression with the presence of this motif was not observed. A possible function in mediating circadian rhythmicity of *Lhc* mRNA accumulation was discussed since a short promoter fragment of the *Lhc b1*1* (*cab2*) gene

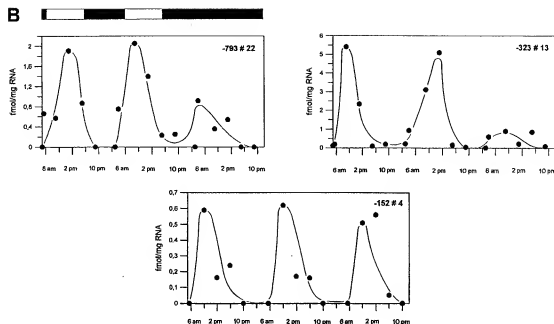


Figure 3. Continued.

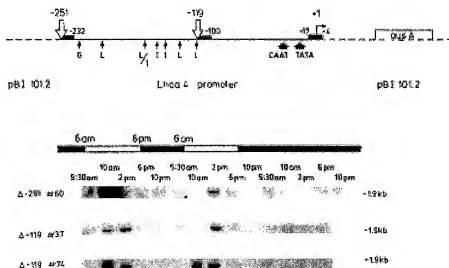


Figure 4. Steady-state mRNA levels of the tomato *Lhc a4* (*cab 11*) gene in transgenic tobacco. The *Lhc a4* promoter regions (-251 to +4 or -119 to +4) were cloned into the pBI 101.2 reporter vector from Clontech. Transgenic tobacco plants containing the constructs were grown in light/dark (light: 06:00–18:00) and continuous dark conditions. Leaves were harvested at indicated time points and the glucuronidase mRNA levels were detected by northern blot analysis. The length of the *gus* mRNA is ca. 1.9 kb. For abbreviations, see Figure 5.

of *Arabidopsis thaliana* containing this motif confers circadian *Lhc* expression in transgenic plants [2]. In addition, the sequence 'ACTT' flanking the 'GATA'

sequence in tomato was suggested to be a component of the signal transduction chain of the circadian clock [6].

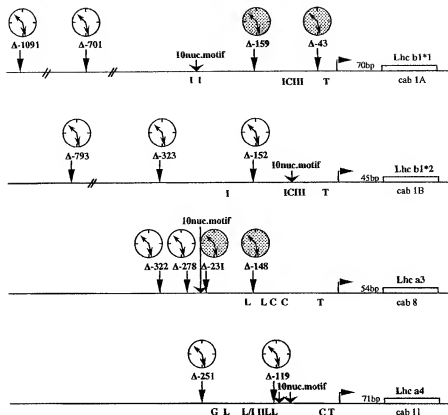


Figure 5. Summary of the deletion analysis. Circadian oscillations of tomato *Lhc* mRNAs in transgenic tobacco are indicated by the 'white' clock. No rhythmic *Lhc* mRNA accumulations were detected in tobacco lines transformed with the tomato *Lhc b1*1* Δ-159 and Δ-43 and the *Lhc a3* Δ-231 and Δ-148 deletion constructs and are indicated by the 'shaded' clock. T, 'TATA'; C, 'CCAAAT'; L, 'GATA'; L, 'CCTTATCAT'; G, 'ACGT'; 10nuc.motif, 'CCANNNNATC'.

Since all members of the tomato *Lhc* gene family express the typical circadian mRNA accumulation pattern a computer-based search was initiated to screen for a conserved *cis*-regulatory element in the 5'-upstream sequences. This analysis was performed with long DNA sequences (e.g. 400 nucleotides) but failed to identify a possible candidate which may be responsible for the mRNA oscillations [25]. Therefore the primary goal of the presented experiments was to explore the 5'-upstream sequences of the tomato *Lhc* genes by promoter deletion analysis in transgenic tobacco plants.

Promoter deletion analysis

Tobacco leaf discs were transformed via *Agrobacterium tumefaciens*-mediated gene transfer with promoter deletion constructs of four tomato *Lhc* genes,

*Lhc b1*1* (*cab 1A*; accession numbers M14445, M30616; promoter: X60922), *Lhc b1*2* (*cab 1B*, M14443; promoter: X60923), *Lhc a3* (*cab 8*; X15258), *Lhc a4* (*cab 11*, X57706). About fifty primary transformants of each deletion construct were regenerated and tested for *Lhc* mRNA expression levels. Individual plant lines with high expression levels were chosen for further analysis.

To find out which of the deleted promoters mediate the circadian oscillations of tomato *Lhc* mRNA, the tobacco transformants were grown in LD and DD conditions. Leaves were harvested at appropriate time points and steady-state mRNA levels were determined by northern blot (Figure 4 for a description of methods, see [24]) or primer extension (Figures 1A, 2, 3A) analysis and quantitated by primer extension analysis (Figure 1B and 3B, for a description of methods, see

Table 1. Sequence motif presence in 5'-upstream regions of *Lhc* genes.

<i>Lycopersicon esculentum</i>	(-701 to -159)	<i>Lhc</i> b1*1	-254	CAAAGATATC
	(-152 to -1)	<i>Lhc</i> b1*2	-87	CAATGAGATC*
	(-278 to -231)	<i>Lhc</i> a3	-237	CAAGAGTATC
	(-119 to -1)	<i>Lhc</i> a4	-116	CAACTCAATC
<i>Triticum aestivum</i>	(211 to -90)	<i>Lhc</i> b1	-92	CAAAAAATC
			-176	CAAGAGTATC
			-140	CAATGGCATC
<i>Arabidopsis thaliana</i>	(-111 to -74)	<i>Lhc</i> b1*1	-103	CAAAAAATC
			-87	CAATGAATGA*
	(-900 to -1)	<i>Lhc</i> b1*2	-371	CAATGGAATC
			-253	CAATGAAGTTATC
			-174	CAATGAAAAATC
			-106	CAAAATC

*Sequence overlaps with the 'CAAT' box.

[18, 25]. The mRNA levels of the tomato *Lhc* b1*1 deletions Δ -1091 (Nos. 4, 11, 38) and Δ -701 (Nos. 4, 9, 26) clearly oscillate in LD with a period of ca. 24 h. Only very little or no tomato *Lhc* mRNA is detectable at 06:00, maximum mRNA levels were reached at 10:00 and levels decrease thereafter (Figure 1A and B). The phase of the circadian rhythms are not altered in different deletion constructs. In constant darkness the amplitudes are significantly reduced but transcripts increase after the night trough at the appropriate time may indicate that an endogenous oscillator influences the expression of the tomato *Lhc* gene.

In contrast to the long promoter constructs it is very unlikely that the tobacco lines with the short promoter regions of *Lhcb1**1 Δ -159 (Nos. 2, 42, 47) and Δ -43 (Nos. 24, 28, 33, 34) exhibit circadian mRNA accumulation patterns (representative data in Figure 1). The mRNA levels fluctuate only twofold at low expression levels, however a constant period length of ca. 24 h was not measured in LD and DD, particularly well documented after quantitation (Figure 1B, right panels). These results clearly show that the short 5'-upstream regions are sufficient for a basal mRNA accumulation and strongly suggests that sequences upstream of -159 are necessary for circadian mRNA accumulation of the tomato *Lhc* b1*1 gene. Furthermore, it is interesting to note that the expression level of the short constructs do not decrease in DD, while this is usually observed for complete *Lhc* genes which are in their native 3' and 5' nucleotide surrounding.

With the *Lhc* a3 deletion constructs similar results were obtained as with *Lhc* b1*1 (Figure 2). Deletion Δ -322 (No. 6) and Δ -278 (No. 14) exhibited circa-

dian *Lhc* mRNA accumulation while the mRNA of the deletion constructs Δ -231 (Nos. 24, 36) and Δ -148 (Nos. 16, 25) reached almost constant levels in LD and DD and no oscillations with a defined period length could be observed. Based on these data it is likely that a region of 47 nucleotides (-278 to -231) is necessary for circadian *Lhc* a3 mRNA oscillations. The low expression levels of the *Lhc* a3 gene in transgenic tobacco prevented a quantitation with the primer extension analysis.

Investigation of the *Lhc* b1*2 (Figure 3) and *Lhc* a4 (Figure 4) genes in tobacco revealed circadian *Lhc* mRNA accumulations for constructs that contain long as well as short 5'-upstream regions (*Lhc* b1*2: Δ -793, Nos. 11, 22; Δ -323, Nos. 13, 17; Δ -152, Nos. 1, 4, 21, 24, *Lhc* a4: Δ -251, No. 60, Δ -119, No. 37, 74). The mRNA constructs of the *Lhc* b1*2 gene could be quantitated with the primer extension analysis and representative results are depicted in Figure 3B. A circadian oscillatory pattern is obvious for each deletion construct. It should be noted that the short 5'-upstream regions of *Lhc* b1*2 and *Lhc* a4 are apparently sufficient to confer circadian rhythmicity. This is a surprise since in contrast such short promoter regions of the *Lhc* b1*1 and *Lhc* a3 genes exhibit an almost constant expression pattern (Figures 1 and 2). It therefore can be concluded that different 5'-upstream regions (relative to the transcription start sites) of the tomato *Lhc* genes are necessary for conferring circadian rhythmicity. The results of all deletion constructs are summarized in an overview in Figure 5.

Sequence comparison

The promoter deletion analysis indicated regions which are necessary for the transmission of the circadian clock in transgenic plants. If a conserved 'clock' regulatory element exists it should be localized within these respective regions. Therefore the following 5'-upstream sequences were aligned: *Lhc* b1*1, -701 to -159; *Lhc* b1*2, -152 to -1; *Lhc* a3, -278 to -231; *Lhc* a4, -119 to -1. A novel sequence motif appears to be conserved in all four tomato *Lhc* 5'-upstream regions (Table 1). The sequence is 'CAANNNNATC', it is composed of three conserved nucleotides, four variable nucleotides followed by three conserved nucleotides. Identical or very similar motifs are present in short 5' promoter regions of circadian controlled *Lhc* genes of *Arabidopsis thaliana* (*Lhc* b1*1 (*cab* 2), -111 to -74 [2, 7]; *Lhc* b1*2 (*cab* 3), -371 [23] and *Triticum aestivum* (*Lhc* b1 (*cab* 1), -211 to -90 [10]). Consistent with the hypothesis that this motif plays a role in transmitting circadian rhythmicity is its absence in the promoter of the non-circadian expressed *Lhc* b gene of *Pinus contorta* (-1000 to +1 [5]). Also interesting in this context are the investigations of Wang *et al.* [30]. Following the phytochrome-regulated light signal transduction pathway of the *A. thaliana* *Lhc* b1*3 gene a specific protein, CCA1, a myb-related transcription factor, was identified. Interestingly, the binding site of this transcription factor overlaps with the motif which we found to be conserved in the 5'-upstream regions that are necessary for circadian expression of the four tomato *Lhc* genes. These findings support the emerging idea that light perception and clock function are closely related, a hypothesis recently stated by Kay [17]. His conclusion was based on the presence of the PAS domains in clock-related proteins such as PER (period, *Drosophila*) and WC (white collar, *Neurospora*) as well as in photosensory proteins such as phytochrome (PHY, plants) and the bacterial blue-light receptor (PYP, photoactive yellow protein [20]). Isolation and characterization of the *trans*-regulatory factor(s) binding to the novel 'CAANNNNATC' motif are on the way and will bring further knowledge regarding the hypothesis that the signal transduction pathways of the light and the circadian clock cross-talk or converge.

Acknowledgements

We thank Dr E. Pichersky (Ann Arbor, MI) for providing us with the tomato *Lhc* a3 and *Lhc* a4 genomic clones and unpublished promoter sequences. Thanks are also due to Dr U. Sonnewald and co-workers (Gatersleben, Germany) who performed the plant transformations. We thank Mrs U. Schäfer for the deletion constructs of *Lhc* a3, Mr D. Dreesmann for initial investigations of some transgenic lines and M. Nolte for sequencing the promoter of *Lhc* b1*1 of tomato. B.P. was supported by the DFG (153/8-2).

References

- Anderson JM, Andersson B: The dynamic photosynthetic membrane and regulation of photosynthesis. *Trends Biochem Sci* 13: 351-355 (1988).
- Anderson SL, Teakle GR, Martino-Catt SJ, Kay SA: Circadian clock- and phytochrome-regulated transcription is conferred by a 78 bp cis-acting domain of the *Arabidopsis* *cab* 2 promoter. *Plant J* 6: 457-470 (1994).
- Armstrong GA, Weisshaar B, Halbrock K: Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with 'ACGT' cores. *Plant Cell* 4: 525-537 (1992).
- Aronson BD, Johnson KA, Loros JJ, Dunlap JC: Negative feedback defining a circadian clock: Autoregulation of the clock gene frequency. *Science* 263: 1578-1584 (1994).
- Barrett JW, Beech RN, Danilchik BF, Siroock C: A genomic clone of a type I *cab* gene encoding a light harvesting chlorophyll *a/b* binding protein of photosystem II identified from lodgepole pine. *Genome* 37: 166-172 (1994).
- Borello U, Ceccarelli E, Giuliano G: Constitutive, light-responsive, and circadian clock-responsive factors compete for the different L-box elements in plant-regulated promoters. *Plant J* 4: 611-619 (1993).
- Carre IA, Kay SA: Multiple DNA-protein complexes at a circadian-regulated promoter element. *Plant Cell* 7: 2039-2051 (1995).
- Castresana C, Stanoloni R, Malik VS, Cashmore AR: Molecular characterization of two clusters of genes encoding the type I *cab* polypeptides of PSII in *Nicotiana plumbaginifolia*. *Plant Mol Biol* 10: 117-126 (1987).
- Donald RKG, Schindler U, Batschauer A, Cashmore AR: The plant G-box promoter sequence activates transcription in *Saccharomyces cerevisiae* and is bound in vitro by a yeast activity similar to GBF, the plant G-box binding factor. *EMBO J* 9: 1727-1735 (1990).
- Fejes E, Pay A, Kanevsky I, Szell M, Adam E, Kay SA, Nagy F: A 268 bp upstream sequence mediates the circadian clock-regulated transcription of the wheat *cab* 1 gene in transgenic plants. *Plant Mol Biol* 15: 921-932 (1990).
- Foster R, Izawa T, Chua NH: Plant bZIP proteins gather at 'ACGT' elements. *FASEB J* 8: 192-199 (1994).
- Gidoni D, Brosio P, Bond-Nutter D, Bedbrook J, Dunsmuir P: Novel cis-acting elements in *Petunia* *cab* gene promoters. *Mol Gen Genet* 215: 337-344 (1994).

13. Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR: An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. *Proc Natl Acad Sci USA* 85: 7089-7093 (1988).
14. Grob U, Stüber K: Discrimination of phytochrome-dependent, light-inducible from non-light-inducible plant genes. Prediction of a common light-responsive element (LRE) in phytochrome-dependent, light-inducible plant genes. *Nucl Acids Res* 15: 9957-9973 (1987).
15. Izawa T, Foster R, Chua NH: Plant bZIP protein binding specificity. *J Mol Biol* 230: 1131-1144 (1993).
16. Jansson S: The light-harvesting chlorophyll *a/b* binding proteins. *Biochim Biophys Acta* 1184: 1-19 (1994).
17. Kay S: PAS, present, and future: clues to the origins of circadian clocks. *Science* 276: 753-754 (1997).
18. Kellmann JW, Merforth N, Wiess M, Pichersky E, Piechulla B: Coordinated circadian oscillations in transcript levels of nineteen *Lha/b* (*cab*) genes in *Lycopersicon esculentum* (tomato). *Mol Gen Genet* 237: 439-448 (1993).
19. Kondo T, Strayer CA, Kulkarni RD, Taylor W, Ishiura M, Golden SS, Johnson CH: Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc Natl Acad Sci USA* 90: 5672-5676 (1993).
20. Lagarias D.M., Shu-Hsing W., Lagarias J.C: A typical phytochrome gene structure in the green alga *Metosphaerium caldarium*. *Plant Mol Biol* 29: 1127-1142 (1995).
21. Lam E, Chua NH: ASF-2: A factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in *cab* promoters. *Plant Cell* 1: 1147-1156 (1989).
22. Manzano T, Carrasco P, Gruissem W: Developmental and organ-specific changes in promoter DNA-protein interactions in the tomato *rbc S* gene family. *Plant Cell* 3: 1305-1316 (1991).
23. Millar A, Kay SA: Circadian control of *cab* gene transcription and mRNA accumulation in *Arabidopsis*. *Plant Cell* 3: 541-550 (1991).
24. Piechulla B, Gruissem W: Diurnal mRNA fluctuations of nuclear and plastid genes in developing tomato fruits. *EMBO J* 6: 3593-3599 (1987).
25. Piechulla B, Kellmann JW, Pichersky E, Schwartz E, Förster HH: Determination of steady-state mRNA levels of individual chlorophyll *a/b* binding protein genes of the tomato *cab* gene family. *Mol Gen Genet* 230: 413-422 (1991).
26. Piechulla B: 'Circadian clock' directs the expression of plant genes. *Plant Mol Biol* 22: 533-542 (1993).
27. Riesselmann S, Piechulla B: Diurnal and circadian light-harvesting complex and quinone B-binding protein synthesis in leaves of tomato (*Lycopersicon esculentum*). *Plant Physiol* 100: 1840-1845 (1992).
28. Sassone-Corsi P: Rhythmic transcription and regulatory loops: winding up the biological clock. *Cell* 78: 361-364 (1994).
29. Schindler U, Cashmore AR: Photoregulated gene expression may involve ubiquitous DNA binding proteins. *EMBO J* 9: 3415-3427 (1990).
30. Wang Z-Y, Kenigsbuch D, Sun L, Haral E, Ong M.S, Tobin E.M: A myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis Lhc b* gene. *Plant Cell* 9: 491-507 (1997).
31. Williams ME, Foster R, Chua NH: Sequences flanking the hexameric G-box core 'ACGTTG' affect the specificity of protein binding. *Plant Cell* 4: 485-496 (1992).

EXHIBIT C

Regulation of Root Hair Initiation and Expansin Gene Expression in Arabidopsis^[9]

Hyung-Taeg Cho¹ and Daniel J. Cosgrove

Department of Biology, Pennsylvania State University, 208 Mueller Laboratory, University Park, Pennsylvania 16802

The expression of two Arabidopsis expansin genes (*AtEXP7* and *AtEXP18*) is tightly linked to root hair initiation; thus, the regulation of these genes was studied to elucidate how developmental, hormonal, and environmental factors orchestrate root hair formation. Exogenous ethylene and auxin, as well as separation of the root from the medium, stimulated root hair formation and the expression of these expansin genes. The effects of exogenous auxin and root separation on root hair formation required the ethylene signaling pathway. By contrast, blocking the endogenous ethylene pathway, either by genetic mutations or by a chemical inhibitor, did not affect normal root hair formation and expansin gene expression. These results indicate that the normal developmental pathway for root hair formation (i.e., not induced by external stimuli) is independent of the ethylene pathway. Promoter analyses of the expansin genes show that the same promoter elements that determine cell specificity also determine inducibility by ethylene, auxin, and root separation. Our study suggests that two distinctive signaling pathways, one developmental and the other environmental/hormonal, converge to modulate the initiation of the root hair and the expression of its specific expansin gene set.

INTRODUCTION

Root hairs are polarized outgrowths of root epidermal cells. In Arabidopsis, root hairs normally arise from epidermal cells that contact two underlying cortical cells (the so-called H position), whereas epidermal cells overlying a single cortical cell (in the N position) develop into nonhair cells (Dolan et al., 1993; Galway et al., 1994). This position-dependent hair cell differentiation thus results in a striped pattern of hair cell files along the long axis of the root, which is found in members of Brassicaceae and in a few species of other families (Cormack, 1947; Dolan and Costa, 2001). Root hair development in Arabidopsis can be divided into three phases: cell specification, initiation, and elongation. Cell specification refers to the fate determination of epidermal cells into hair cells and nonhair cells, depending on position. Initiation refers to the formation of a protrusion or bulge at the site of hair outgrowth. Elongation refers to the process of sustained tip growth that normally follows initiation. Numerous experimental observations indicate that these three phases involve different cellular and genetic processes (for reviews, see Schiefelbein, 2000; Foreman and Dolan, 2001).

Several genes that control root epidermal cell specification have been identified. Loss-of-function mutations in TTG (TRANSPARENT TESTA/GLABROUS) or GL2 (GLABRA2) result in root hairs in both H and N positions (Galway et al., 1994; Masucci et al., 1996), indicating that TTG (a protein with WD40 repeats) and GL2 (a homeodomain transcription factor) function as negative regulators of the differentiation of nonhair cells to hair cells. Mutations in another MYB transcription factor, WER (WEREWOLF), also generate root hairs in almost every root epidermal cell, because WER positively regulates GL2 expression (Lee and Schiefelbein, 1999). On the other hand, *cpc* (*caprice*) mutants have only a few root hairs, indicating that CPC, a MYB-like protein, functions as a positive regulator for root hair cell differentiation (Wada et al., 1997). A recent study demonstrated the interactions among these regulatory genes (Lee and Schiefelbein, 2002). In the N position, WER positively regulates the expression of CPC and GL2. CPC (or its downstream signal) appears to move to cells in the H position and inhibits the expression of WER, CPC, and GL2, which leads the cell to initiate hair formation.

Root hair initiation, which is genetically downstream of GL2 (Masucci and Schiefelbein, 1996), is regulated by another set of genes and is sensitive to hormonal and environmental factors (Schiefelbein, 2000). The auxin-resistant mutant (*axr2*) develops few root hair bulges (Wilson et al., 1990), and the defect of root hair initiation in root hair defective (*rhda6*) can be reversed by treatment with auxin or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid

¹To whom correspondence should be addressed. E-mail hxc31@psu.edu; fax 814-865-9131.

^[9]Online version contains Web-only data.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.006437.

(ACC) (Masucci and Schiefelbein, 1994, 1996). ACC treatment of wild-type plants induces root hairs in the N position, as do the constitutively ethylene-responsive *ctr1* and ethylene-overproducing *eto* mutants (Dolan et al., 1994; Masucci and Schiefelbein, 1996; Cao et al., 1999). ACC has been suggested as a factor that determines the developmental fate of cells in the H position (Tanimoto et al., 1995). Also implicating ethylene involvement in root hair initiation, the ethylene biosynthesis inhibitor aminooxyvinylglycine (AVG) and silver ion (an inhibitor of ethylene perception) have been found to inhibit root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995). However, the role of ethylene in root hair formation is questioned because the ethylene-insensitive mutants *etr1* and *eln2* maintained normal root hair numbers (Masucci and Schiefelbein, 1996). Additionally, environmental factors such as nutrients (Peterson and Stevens, 2000), light, and separation of the root from the agar medium (Okada and Shimura, 1994) also affect root hair development. It has been suggested that hormones and environmental factors affect root hair initiation through a pathway distinctive from the normal development-associated pathway (Okada and Shimura, 1994; Schiefelbein, 2000), but experimental confirmation for this is needed.

Elongation of the root hair is achieved by tip growth (Schiefelbein, 2000). Hair elongation likely is governed by genetic components distinct from those that govern hair initiation, but root hair elongation is influenced by auxin, ethylene, and environmental factors as well (Okada and Shimura, 1994; Pitts et al., 1998; Schiefelbein, 2000).

Spatial regulation of cell wall expansion is critical for cell morphogenesis in plants (Fowler and Quatrano, 1997). Thus, outgrowth of the root hair from the epidermal cell is expected to accompany localized cell wall loosening at the correct position. Bibikova et al. (1998) demonstrated localized wall acidification at the site of root hair initiation. This acidification could activate expansins. Expansins are cell wall-loosening proteins capable of mediating cell wall extension in acidic conditions without hydrolytic breakage of major structural components of the cell wall (McQueen-Mason et al., 1992; for recent reviews, see Cosgrove, 2000; Lee et al., 2001). Expansin genes are found throughout the entire plant kingdom (Cosgrove, 1999; Li et al., 2002), and their pattern of expression indicates that they are related closely to cell growth and tissue differentiation (for review, see Cho, 2001). Alteration of endogenous expansin gene expression modulates leaf growth and pedicel abscission in *Arabidopsis* (Cho and Cosgrove, 2000) and leaf morphology and phyllotaxy in tobacco (Pien et al., 2001). Two families of expansins are recognized at present (Cosgrove, 2000), α - and β -expansins, and *Arabidopsis* has 26 α - and 5 β -expansin genes (see <http://www.bio.psu.edu/expansins>). In the course of analyzing the expression of these genes in *Arabidopsis*, two α -expansin genes, *AtEXP7* and *AtEXP18*, were found to be expressed specifically in root hair cells (D.M. Durachko and D.J. Cosgrove, unpublished data).

In this study, we examined in detail the expression patterns of these two root hair-specific expansin genes in various root hair mutants as well as under hormonal (auxin and ethylene) and environmental (separation of the root from the medium) treatments. In particular, the role of endogenous ethylene in root hair development was studied closely. Promoter analyses of the two expansin genes, in conjunction with the effect of root hair-inducing factors, also were conducted to elucidate the regulation of expression of these root hair-specific genes. Our results show that the expression of these expansin genes is linked tightly to root hair initiation and subsequent elongation. Moreover, we find that, although ethylene mediates the effects of auxin and root separation on root hair development, it is not essential for the normal (or default) development of root hairs in wild-type plants. These results alter current views of ethylene involvement in root hair development.

RESULTS

Root Hair Cell-Specific Expression of *AtEXP7* and *AtEXP18*

RNA gel blot and promoter-reporter gene expression analyses were performed to investigate the organ- and tissue-specific expression patterns of *AtEXP7* and *AtEXP18*. The transcripts of both expansin genes were found in the root but were undetectable in other major plant organs (Figure 1). Wild-type plants harboring the *AtEXP7* promoter: β -glucuronidase (*GUS*) or *AtEXP7* promoter:green fluorescent protein (*GFP*) construct showed staining (or fluorescence) solely

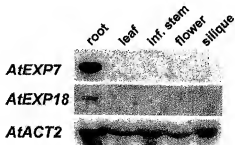


Figure 1. Expression of *AtEXP7* and *AtEXP18* in Different Tissues.

Total RNA was isolated from seedling roots, young leaves, growing inflorescence (inf.) stems, whole floral organs, and young green siliques of *Columbia* wild-type *Arabidopsis* plants. Twenty micrograms of total RNA was analyzed per lane. The transcript levels of *Arabidopsis* actin2 (*AtACT2*) served as a loading control.

in root hair cell files (Figures 2A to 2D). No reporter gene expression was found in other cell types of the root or other organs except a weak expression in the inner layer of the seed coat. Different ecotypes, Columbia and Wassilewskija, showed the same reporter gene expression pattern. The expression of *AtEXP7* occurred approximately one cell before the root hair bulges appeared (Figure 2B), indicating the gene's close temporal expression with the hair initiation process. Plants harboring the *AtEXP18* promoter::reporter construct also showed the same expression pattern as plants with the *AtEXP7* promoter::reporter construct (data not shown). However, the level of *AtEXP18* expression was lower than that of *AtEXP7*. Promoter analyses, as described below, showed that the average promoter activity of *AtEXP18* was ~60% of *AtEXP7* promoter activity. In this study, the expression pattern of *AtEXP7* is described in greater detail, but the results also hold for *AtEXP18*.

The *AtEXP7* protein expression pattern also was examined by expressing the *AtEXP7*-GFP fusion protein driven by the *AtEXP7* promoter. The cell-type specificity and the timing of protein expression were almost identical with the expression pattern of the reporter gene alone (Figures 2E to 2G). The fluorescence from the fusion protein was highest in regions of root hair initiation and elongation. Although the *AtEXP7*-GFP fusion protein tended to localize more at the emerging root hair tip and to distribute peripherally in the root hair cell (Figures 2E to 2G), it was detected predominantly inside the plasma membrane upon plasmolysis (data not shown). This finding indicates that the fusion protein was not secreted to the cell wall.

We have searched for mutants defective in *AtEXP7* or *AtEXP18*. An Arabidopsis line that includes a T-DNA insertion in the second intron of *AtEXP7* was identified, but the homozygous line still expressed transcripts of the correct size, albeit at a lower level than in the wild type. This line did not show obvious alterations in the root hair, most likely as a result of the leakiness of the mutation and functional redundancy by *AtEXP18* and perhaps other expansin genes.

Effect of Root Hair-Regulating Factors on the Expression of Root Hair Expansin Genes

Root hair formation in Arabidopsis is regulated by developmental regulators, hormones, and environmental factors. Because *AtEXP7* is a root hair-specific gene and is thought to function in root hair formation, we investigated whether *AtEXP7* expression is modulated by various root hair-regulating factors. For this purpose, the *AtEXP7* promoter::GUS reporter construct was introduced into root hair mutants, and the reporter gene expression pattern was monitored.

In *ttg* and *gl2* mutants, which have hairs in both the H and N positions, *AtEXP7* promoter::GUS was expressed in both positions (Figures 2H and 2I), suggesting that TTG and GL2 negatively regulate the expression of *AtEXP7*, just as they

negatively regulate root hair formation in the N position of the wild-type plant (Galway et al., 1994; Masucci et al., 1996).

The *axr2* mutant is defective in hair elongation and partially in hair initiation; thus, it produces few root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Figure 2J). The spatial pattern of *AtEXP7* promoter::GUS expression was not changed in this mutant (Figure 2J) compared with that in wild-type plants. However, the expression level of *AtEXP7* was much lower in the mutant than in the wild type (Figure 3). Because auxin positively regulates root hair formation and *AtEXP7* expression (see below), AXR2 likely downregulates the expression of *AtEXP7* and partially inhibits root hair formation.

The mutant *rhb6* also is defective in root hair initiation, but unlike *axr2*, it develops almost no root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Figure 2K). *AtEXP7* expression in *rhb6* was blocked almost completely, as shown by GUS expression and by transcript analysis (Figures 2K and 3). In *rhb6*, treatment with the ethylene precursor ACC or auxin, or separation of the root from the agar medium induced normal root hair formation (Masucci and Schiefelbein, 1994, 1996) (Table 1). In agreement with their effects on root hair formation, all of these treatments induced *AtEXP7* expression in *rhb6* roots (Figures 2L to 2N and 3). *AtEXP18* expression in *rhb6*, as described below, also was inducible by these treatments. These results indicate that RHB6 is a positive regulator of *AtEXP7* and *AtEXP18* expression.

Exogenous Ethylene Is a Positive Effector for the Expression of Root Hair Expansin Genes in Concert with Root Hair Formation

Because ethylene is a positive effector of root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995), we investigated whether ethylene coordinately regulates the expression of root hair expansin genes with root hair formation. The ethylene precursor ACC (5 μ M) induced root hair formation and *AtEXP7* expression in the N position of the wild-type root (Figure 2O). Mutation in CTR1, which showed constitutive ethylene effects and thus induced the formation of root hairs in the N position (Table 1), likewise activated *AtEXP7* expression in root hairs in the N position (Figure 2R) and increased the transcript level by 36% relative to that of the wild type (Figure 3). Compared with the wild type, the root hair-defective *rhb6* mutant had only ~10% of the *AtEXP7* transcript (Figure 3), which could derive from the occasional root hairs in the *rhb6* root. Treatment of the mutant with 5 μ M ACC restored 78% of the transcript level and 74% of the root hair number (Table 1). Ethylene gas (1 μ L/L) treatment also induced a similar level of root hairs in the *rhb6* root, as did 5 μ M ACC (data not shown), and the effect of exogenous ethylene or ACC could be blocked completely by 1-methylcyclopropene (1-MCP), the competitive inhibitor of ethylene binding to the

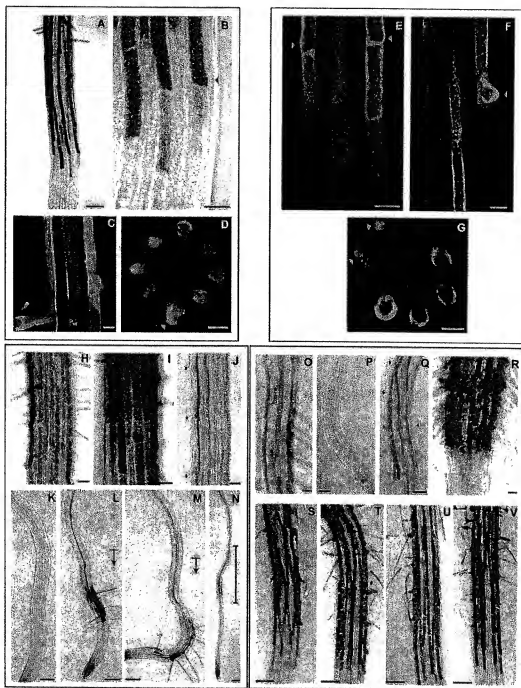


Figure 2. Root Hair Cell-Specific Expression Pattern of *AtEXP7* in the Arabidopsis Root.

(A), (B), and (H) to (V) show *AtEXP7* promoter::GUS expression; (C) and (D) show *AtEXP7* promoter::GFP expression; and (E) to (G) show *AtEXP7* promoter::genomic *AtEXP7*-GFP expression.

(A) to (D) In the wild-type root, reporter gene expression occurs in the root hair cell files. The weaker blue staining between the strong stains are from the hair cell files of the opposite side. (C) shows an optical longitudinal section demonstrating GFP expression at the root hair cell files. The

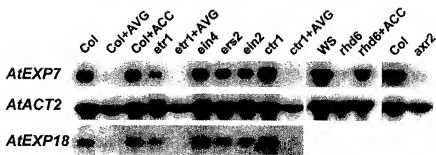


Figure 3. RNA Gel Blot Analyses of *AtEXP7* and *AtEXP18* Transcripts in Different Mutant Backgrounds and under Treatment with Ethylene Precursor and Inhibitor.

Total RNA was prepared from roots of 4-day-old wild-type and mutant seedlings. For ACC (5 μ M) and AVG (5 μ M) treatments, the seedlings were transferred to chemical-containing plates on day 3. Ten micrograms of total RNA, except for Wassilewskija and *rh6* (30 μ g), was analyzed. The transcript level of Arabidopsis actin2 (*AtACT2*) served as a loading control. Col, Columbia wild type; WS, Wassilewskija wild type.

receptors. We chose 1-MCP as an antagonist of ethylene action because of its high specificity of action and lack of deleterious side effects (Sisler et al., 1996; Hall et al., 2000). At 1 μ M/L, 1-MCP almost completely abolished ACC-induced root hair formation and the expression of *AtEXP7* and *AtEXP18* in *rh6* (Figure 4).

1-MCP Inhibits Auxin- or Root Separation-Induced Root Hair Formation and Expression of Root Hair Expansin Genes

To investigate the possible involvement of ethylene receptors in root hair formation and expansin gene expression induced by auxin or root separation from the agar medium,

the antagonism of these effectors by 1-MCP was investigated in the *rh6* background. Auxin- or root separation-induced root hair formation was greatly inhibited by 1-MCP (1 μ M/L). No root hair bulges or elongated root hairs were observed in mutant seedlings treated with indole 3-acetic acid (IAA; 30 nM) together with 1-MCP (Figure 5B). Similarly, 1-MCP inhibited 90% of the root hair formation induced by root separation (Figures 6B and 6C). Consistent with these results, 1-MCP inhibited 70 to 90% of IAA- or root separation-induced expression of *AtEXP7* and *AtEXP18* (Figures 5C to 5H and 6D to 6I). These results show that the coordinate induction of root hairs and expansin gene expression by auxin and root separation requires ethylene sensing, most likely because ethylene is part of the signaling pathway for these effects.

Figure 2. (continued).

red area from propidium iodide indicates the cell boundary. (D) shows an optical cross-section of the root demonstrating gene expression at the eight root hair cells. The arrowheads in (B) and (D) indicate emerging root hair bulges.

(E) to (G) Expression of the *AtEXP7*-GFP fusion protein shows the same pattern as expression of GUS or GFP alone. (G) shows an optical cross-section. Arrowheads indicate emerging root hair bulges.

(H) and (I) in the *ttg-1* (H) and *g2-1* (I) backgrounds, reporter gene expression is observed in cells from both the H and N positions.

(J) *axr2-1* background. Arrowheads indicate some root hair bulges.

(K) to (N) *rh6* background with no treatment (K) or with 5 μ M ACC (L), 30 nM IAA (M), or separation of the root from the medium (N). The bases of the arrows in (L) and (M) indicate the approximate starting points of hormone treatments. The vertical bar in (N) indicates where the root was separated from agar.

(O) to (Q) Wild-type roots treated with 5 μ M ACC (O), 5 μ M AVG (P), or 50 μ M silver ion (Q). Stars in (O) indicate ectopic expression of GUS in the N positions.

(R) *ctr1-1* background. Stars indicate ectopic expression of GUS in the N positions.

(S) to (U) Dominant ethylene receptor mutants *etr1-1* (S), *ein4* (T), and *ers2-1* (U).

(V) *ein2-1* background.

Bars = 100 μ m in (K) to (N), 50 μ m in (A), (P), (Q), and (S) to (V), and 20 μ m in (B) to (J), (O), and (R).

Table 1. Root Hair Number in Wild-Type and Mutant Plants with Ethylene Precursor or Inhibitor Treatment

Plant	Percent of Total Root Hair Cells ^a				Percent of Root Hair Cells in the N position ^b	
	No Treatment	ACC (5 μ M)	AVG (5 μ M)	1-MCP (1 μ L/L)	No Treatment	ACC (5 μ M)
<i>Columbia</i>	51.1 \pm 3.3	65.9 \pm 5.8	1.3 \pm 2.3	44.0 \pm 4.2	1.1 \pm 3.3	15.9 \pm 5.8
<i>etr1-1</i>	45.8 \pm 6.7	48.1 \pm 3.7	0 \pm 0	43.0 \pm 2.7	2.7 \pm 3.3	2.5 \pm 4.6
<i>etr2</i>	51.3 \pm 2.3	51.5 \pm 3.4	0.6 \pm 1.8	48.3 \pm 2.5	1.3 \pm 2.3	1.5 \pm 3.4
<i>ers1</i>	55.4 \pm 6.2	52.5 \pm 4.2	0.6 \pm 1.8	49.5 \pm 2.7	5.4 \pm 6.2	2.5 \pm 4.2
<i>ers2-1</i>	50.0 \pm 3.0	55.0 \pm 8.2	0 \pm 0	44.1 \pm 3.8	0.8 \pm 1.9	6.4 \pm 8.5
<i>ein4</i>	50.4 \pm 1.4	57.2 \pm 8.7	0 \pm 0	42.3 \pm 3.5	0.4 \pm 1.4	7.2 \pm 8.7
<i>ein2-1</i>	48.8 \pm 3.8	45.8 \pm 2.0	0 \pm 0	N.D. ^c	2.5 \pm 3.4	0 \pm 0
<i>ctr1-1</i>	65.0 \pm 6.4	N.D.	0 \pm 0	N.D.	15.0 \pm 6.4	N.D.
<i>eto2</i>	63.8 \pm 4.8	N.D.	0 \pm 0	N.D.	13.8 \pm 4.8	N.D.
<i>etr1-7</i>	50.7 \pm 5.1	N.D.	N.D.	N.D.	0.7 \pm 5.1	N.D.
<i>rhg6</i>	0 \pm 0	37.6 \pm 13.9	N.D.	N.D.	0 \pm 0	3.7 \pm 6.6

Values shown are means \pm SD ($n = 140$ to 260).

^aPercentage of root hair-bearing epidermal cells among total epidermal cells counted, including cells in both the H and N positions.

^bPercentage of root hair-bearing epidermal cells at the N position among total epidermal cells counted.

^cN.D., not determined.

Endogenous Ethylene Is Not Involved in Normal (Default) Root Hair Formation and Expression of Root Hair Expansin Genes in the Wild Type

To verify the role of endogenous ethylene during root hair formation and expression of root hair expansin genes, we examined the effects of dominant mutations of ethylene receptors and inhibitors of ethylene action. Here, we use the term "endogenous ethylene" to designate the internal ethylene level in the plant without any mutations or treatments that would induce the overproduction of ethylene.

Our results showed that mutations in the ethylene signaling pathway failed to inhibit root hair formation and expansin gene expression. None of the five dominant-negative ethylene receptor mutants showed a significant reduction in root hair density (Table 1). The *ein2* mutant, which is known to exhibit the strongest ethylene phenotype, also had a normal number of root hairs, consistent with a previous report (Masucci and Schiefelbein, 1996). *AtEXP7* expression also was patterned normally in roots of the ethylene mutants (Figures 2S to 2V), and expression levels were not reduced greatly in the mutant backgrounds (Figure 3).

Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, has been used to test the role of ethylene in root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995). Our results showed that 5 μ M AVG almost completely blocked root hair formation and *AtEXP7* expression in the wild type (Figures 2P and 3, Table 1). However, surprisingly, AVG (5 μ M) almost completely inhibited root hair formation in the constitutively ethylene-responsive mutant *ctr1-1* (Table 1), even though this mutant should not respond to AVG inhibition of ethylene synthesis.

AVG markedly repressed the expression of *AtEXP7* in *ctr1-1* and other genotypes, but it also reduced actin gene expression (*AtACT2*; Figure 3). Although it was reported that ACC could partially restore root hair formation in the AVG-treated root (Masucci and Schiefelbein, 1994, 1996), our results indicate that AVG has significant deleterious effects on root hair development. Toxicity of AVG also is reported in root formation (Jackson, 1991) and somatic embryogenesis (Meijer, 1989). This may occur because AVG, functioning as an inhibitor of pyridoxal phosphate-dependent enzymes (Abel, 1985), probably interferes with other biochemical processes that are vulnerable to the inhibitor, not only ethylene biosynthesis.

To further test the role of endogenous ethylene during root hair formation in wild-type plants, we used 1-MCP, which binds to multiple ethylene receptors (Hall et al., 2000). Thus, we expected that 1-MCP would strongly inhibit root hair formation in wild-type plants if endogenous ethylene were involved. However, root hair formation in the wild-type root was inhibited very little by 1 μ L/L 1-MCP (Table 1) or even by 10 μ L/L (data not shown). By contrast, 0.22 μ L/L 1-MCP showed saturated inhibitory effects on both ethylene binding to the receptors and the triple response (Hall et al., 2000). 1-MCP also did not significantly inhibit the expression of *AtEXP7* and *AtEXP18* in the wild type (Figure 7). 1-MCP is not able to reverse the constitutive ethylene-responsive phenotype of *ctr1* (Hall et al., 2000), in contrast to the deleterious effect of AVG on the *ctr1* root.

Silver ion (an inhibitor of ethylene perception) at 50 μ M did not abolish root hair formation and *AtEXP7* expression, although it completely inhibited hair elongation (Figure 2Q). A previous study reported that silver ion (1 μ M) greatly reduced root hair number (Tanimoto et al., 1995),

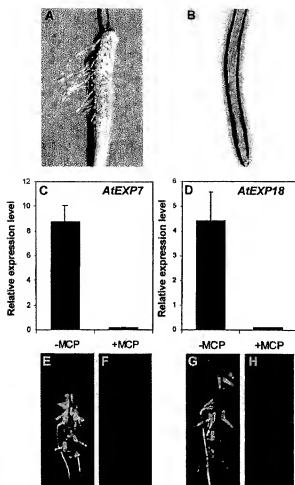


Figure 4. Effect of 1-MCP on ACC-Induced Root Hair Formation and Expansin Gene Expression in the *rhb6* Root.

(A) and (B) Bright-field microscopy images of roots grown in 5 μ M ACC without (A) or with (B) 1 μ L 1-MCP. (C) and (D) Relative expression levels of *AtEXP7* (C) and *AtEXP18* (D) in the root when induced by 5 μ M ACC without (-MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors ($n = 11$ to 18). (E) to (H) Confocal microscopy images of the roots harboring *AtEXP7* promoter::GFP [(E) and (F)] and *AtEXP18* promoter::GFP [(G) and (H)]. Seedlings were incubated in 5 μ M ACC without [(E) and (G)] or with [(F) and (H)] 1-MCP.

but it is not clear whether small bulges were counted. The effects of ACC, AVG, and mutations in ethylene signaling on the expression of *AtEXP18* also resembled those on *AtEXP7* expression, as shown by RNA gel blot analysis (Figure 3).

Endogenous Ethylene Affects Root Hair Elongation

In contrast to root hair initiation, ethylene showed an unambiguous effect on root hair elongation, consistent with a

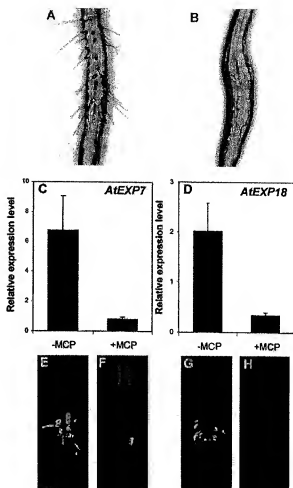


Figure 5. Effect of 1-MCP on IAA-Induced Root Hair Formation and Expansin Gene Expression in the *rhb6* Root.

(A) and (B) Bright-field microscopy images of the roots grown in 30 nM IAA without (A) or with (B) 1 μ L 1-MCP. (C) and (D) Relative expression levels of *AtEXP7* (C) and *AtEXP18* (D) in the root when induced by IAA without (-MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors ($n = 7$ to 12). (E) to (H) Confocal microscopy images of the roots harboring *AtEXP7* promoter::GFP [(E) and (F)] and *AtEXP18* promoter::GFP [(G) and (H)]. Seedlings were incubated in IAA without [(E) and (G)] or with [(F) and (H)] 1-MCP.

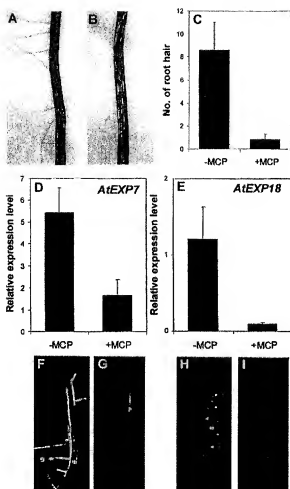


Figure 6. Effect of 1-MCP on Root Separation-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and (B) Bright-field microscopy images of the roots separated from the agar medium without (A) or with (B) 1 μ M 1-MCP. (C) Effect of 1-MCP on root hair number in separation-treated roots. Total root hairs were counted from the separated region of the root. Bars indicate standard errors ($n = 13$ to 19). (D) and (E) Relative expression levels of *AtEXP7* (D) and *AtEXP18* (E) in the root when induced by separation of the root without (-MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors ($n = 11$ to 15). (F) to (J) Confocal microscopy images of roots harboring *AtEXP7* promoter:GFP [(F) and (G)] and *AtEXP18* promoter:GFP [(H) and (I)]. Seedlings whose roots were separated from the medium were incubated without [(F) and (H)] or with [(G) and (I)] 1-MCP.

previous report (Pitts et al., 1998). Root hair length was decreased significantly in four dominant ethylene receptor mutants (Table 2). Treatment with 1-MCP also greatly decreased root hair elongation in the wild type. Considering the effect of each dominant mutation on root hair length, we can assess the cell type-specific roles of the five ethylene receptors. *ETR1* seems to play the most significant role in root hair elongation, followed by $ERS1 \geq ERS2 > ETR2$. *ELN4* appears to have no function in root hair elongation.

Promoter Analyses of *AtEXP7* and *AtEXP18*

AtEXP7 and *AtEXP18* are expressed specifically in the root hair cell and are induced by ethylene, auxin, and separation of the root from the medium. To define the regulatory elements for the hair cell specificity and effector inducibility of the promoter, we performed promoter analyses of the genes by sequential deletion of the 5' regions, nucleotide substitution, and gain of function of the *cis* elements. The deleted or substituted promoters were fused directly to the GFP coding sequence, and the gain-of-function *cis* elements were combined with the 35S minimal promoter region of *Caulliflower mosaic virus* (-64 35S promoter; Eyal et al., 1995) that was followed by the GFP sequence. For unambiguous evaluation of the promoter activities, the promoter:GFP constructs were introduced stably into plants (wild type and *rhd6*) by *Agrobacterium* transformation. To assess the inducibility of promoter activities by ethylene, auxin, and root separation, we treated the transformed *rhd6* plants with 5 μ M ACC, 30 nM IAA, or separation of the root. Promoter activity was evaluated by confocal laser scanning microscopy to measure GFP fluorescence in roots of the first generation of transformants (9 to 62 independent T1 lines per construct, with an average of 28). The histogram function of Adobe Photoshop was used to quantify the relative GFP fluorescence.

For 5' deletion analysis of the *AtEXP7* promoter, sequential deletions from -1380 to +48 bp, relative to the predicted transcription initiation site, were generated (Figure 8A). Deletions to -386 bp did not significantly affect the promoter activity in either the wild type or *rhd6* treated with ACC (Figures 8B and 8C). Further deletion to -245 bp decreased promoter activity by 50 to 70% in both backgrounds, and this level continued through additional deletions to -134 bp. In auxin-treated *rhd6* transformants, the promoter activity decreased gradually in deletions from -386 to -134 bp, where ~50% of the activity remained (Figure 8D). Root separation treatment of *rhd6* also gave a similar result, except that the deletion to -386 bp decreased the promoter activity significantly (Figure 8E).

Although the promoter activity was reduced considerably by deletion to -245 bp, both the cell specificity and the inducibility by effectors were maintained until deletion to -134 bp, and no novel expression patterns were observed in other tissues (data not shown). The cell specificity and the

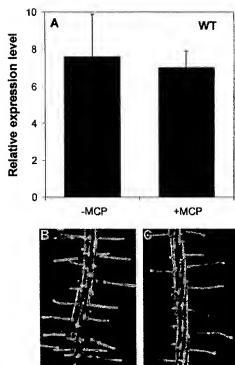


Figure 7. Effect of 1-MCP on Expansin Gene Expression and Root Hair Formation in the Wild Type.

(A) Relative expression levels of *AtEXP7* without (–MCP) or with (+MCP) 1 μ L/L 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter. Bars indicate standard errors ($n = 7$ to 10). WT, wild type. (B) and (C) Confocal microscopy images of roots harboring *AtEXP7* promoter::GFP without (B) or with (C) 1 μ L/L 1-MCP.

Inducibility by effectors disappeared completely with deletion to –70 bp. Although elements for auxin (TGCTC; –808 bp) and ethylene (AATTCAAA; –615 bp) response are located on the *AtEXP7* promoter, deletions of those elements did not affect the responsiveness of the promoter to these hormones (Figures 8C and 8D). Deletion analysis of the *AtEXP7* promoter suggested that the elements for cell specificity and inducibility by these effectors are located between –134 and –70 bp. In this region (Figure 9A) are three repeats of a core binding sequence (AAG) for the DOF zinc finger protein (Yanagisawa and Schmidt, 1999) and one core motif (GGATA) for MYB1, a MYB-like protein (Baranowski et al., 1994). The distal region between –386 and –245 bp likely contains some enhancing elements, because deletion of this region reduced promoter activity significantly. A MYB1 core motif also is found in this distal promoter region (–281 to –276 bp).

To define the *cis*-regulatory elements in the proximal region (–134 to –70 bp) of the *AtEXP7* promoter, seven 9- to 10-bp-long substitution mutations were introduced into this region. To acquire the greatest mutational effects, an A/T base pair was changed to G/C or C/G. Substitution mutations by ~9 to 10 bp are small enough to localize the controlling elements with reasonable precision (Carey and Smale, 2000). These substitutions replaced the DOF and MYB1 core elements and their flanking regions. The substitution mutations E7M1~E7M7 were produced from the –386-bp deletion so that the wild-type promoter had full activity (Figure 9A). Although promoter activity fluctuated between 50 and 130% compared with wild-type (–386 bp) activity, the substitutions E7M1~E7M5 did not greatly diminish promoter activity in either wild-type or ACC-treated *rhod6* roots (Figures 9B and 9C). However, both E7M6 (which includes the MYB1 core element) and E7M7 (flanking E7M6) decreased the activity to ~13 to 26%. Similar results were obtained by treatment with auxin or root separation (data not shown). These results suggest that the 19-bp motif containing the MYB1 element (hereafter called the –80/–62 element) is most important for both hair cell specificity and inducibility by ethylene, auxin, and root separation.

A gain-of-function analysis was performed to confirm that the identified elements are able, in isolation, to direct hair cell specificity. E7G1~E7G3 are short sequences that contain the proximal MYB1 core with different 3' extensions, and E7G4 includes the entire proximal region between –134 and –46 bp. E7G4M6 and E7G4M7 are the same as E7G4 except that they harbor E7M6 and E7M7 substitution mutations, respectively (Figure 9A). The results shown in Figures 9D to 9G are from the wild-type background, but similar results were obtained with ACC-treated *rhod6* (data not shown). The 35S minimal promoter (mp35S) alone did not show GFP expression (Figure 9E). The gain-of-function promoter constructs E7G1~E7G3 showed promoter activity

Table 2. Root Hair Length in Wild-Type and Ethylene Mutant Plants with Ethylene Precursor or Inhibitor Treatment

Plant	No Treatment	ACC (5 μ M)	1-MCP (1 μ L/L)
<i>Columbia</i>	0.91 \pm 0.22	1.07 \pm 0.14	0.51 \pm 0.19
<i>ctr1-1</i>	0.26 \pm 0.14	0.21 \pm 0.20	0.27 \pm 0.16
<i>etr2</i>	0.72 \pm 0.20	0.66 \pm 0.06	0.65 \pm 0.09
<i>ers1</i>	0.40 \pm 0.14	0.41 \pm 0.12	0.39 \pm 0.13
<i>ers2-1</i>	0.47 \pm 0.20	0.47 \pm 0.19	0.35 \pm 0.11
<i>ein4</i>	0.92 \pm 0.12	0.95 \pm 0.12	0.56 \pm 0.13
<i>ein2-1</i>	0.04 \pm 0.02	0.05 \pm 0.03	N.D.*
<i>ctr1-7</i>	0.82 \pm 0.07	N.D.	N.D.
<i>ctr1-1</i>	1.22 \pm 0.18	N.D.	N.D.

Values shown are means \pm SD in mm ($n = 35$).

*N.D., not determined.

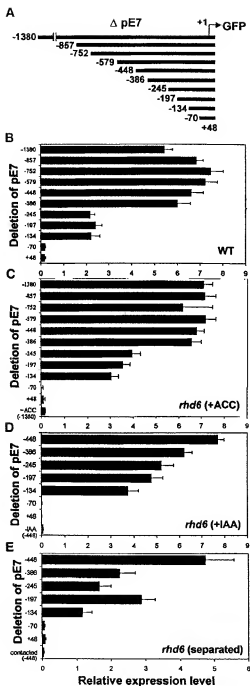


Figure 8. Deletion Analysis of the *AtEXP7* Promoter.

(A) Deletions of the *AtEXP7* promoter ($\Delta pE7$) that are fused to the coding region of GFP. Numbers indicate nucleotide positions relative to the transcription initiation site (+1).

(B) Relative activities (GFP expression) of the truncated *AtEXP7* promoters in the wild-type (WT) root. Bars indicate standard errors.

as weak as that of *mp35S* (Figure 9D), but 20 to 30% of T1 lines from these constructs showed very low and irregular GFP fluorescence in root hair cells (see supplemental data online), which was undetectable in *mp35S* roots. No significant differences in promoter strength among *E7G1*~*E7G3* were found. By contrast, *E7G4* could direct strong hair cell-specific expression of the reporter gene (Figures 9D and 9F). However, the substitution mutation of the *E7G4* promoter fragment at the *E7M6* or *E7M7* site eliminated the promoter activity almost completely (Figures 9D and 9G). This gain-of-function promoter analysis demonstrates that the -80/-62 element confers the hair cell specificity of the *AtEXP7* promoter. However, some additional elements in the proximal region, particularly between -134 and -81 bp, seem to be required for strong promoter activity. These additional elements could be functionally redundant, because the individual substitution mutations (*E7M1*~*E7M5*) elsewhere than in the -80/-62 region did not reduce promoter activity substantially (Figures 9B and 9C).

For analysis of the *AtEXP18* promoter, deletions between -1016 and +42 bp were generated (Figure 10A). The *AtEXP18* promoter activity maintained its full strength until the deletion to -241 bp and showed an ~50% decrease by further deletion to -196 bp in both wild-type and ACC- or IAA-treated *rhd6* seedlings (Figures 10B to 10D). The deletions beyond -145 bp completely eliminated the promoter activity in both the wild type and *rhd6* with effector treatments. A similar change of promoter activity was observed in root separation-treated *rhd6*, except that the deletion to -321 bp reduced the activity significantly (Figure 10E). Deletion analysis of the *AtEXP18* promoter indicated that the -196/-145 region contains elements for hair cell specificity and the -241/-196 region may include some enhancing elements relevant to promoter strength. The -196/-145 region of *AtEXP18*, resembling the -80/-62 element of *AtEXP7*, is likely the target of signals from ethylene, auxin, and root separation, because these effectors all require this region for gene induction (Figure 11). However, the -196/-145 region of *AtEXP18* does not contain the MYB11 binding motif or similar sequences found in the -80/-62 element of *AtEXP7*. This difference indicates that the cell specificity of the two promoters probably is determined by different transcription factors, which nevertheless are regulated similarly by developmental factors, auxin, ethylene, and root separation.

(C) to (E) Relative activities of the truncated *AtEXP7* promoters in the *rhd6* root. For gene induction, the transformed mutant seedlings were treated with 5 μ M ACC (C) or 30 nM IAA (D) or roots were separated from agar to expose them to air (E) for 1 day before observation. Bars indicate standard errors.

In (B) to (E), $n = 27$ to 62.

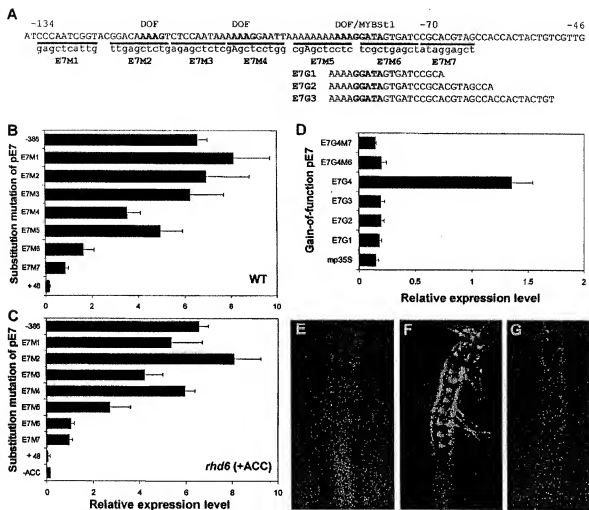


Figure 9. Substitution and Gain-of-Function Analyses of the *AtEXP7* Promoter.

(A) The proximal promoter region of *AtEXP7* between -134 and -46 bp. For substitution mutations (E7M1~E7M7), the underlined regions were replaced by the nucleotides shown in lowercase letters. These substitution mutations were generated from the region between -396 and +48 bp. E7G1~E7G3 are the gain-of-function promoter fragments. The substituted promoters were fused to the coding region of GFP, and the gain-of-function promoter fragments were connected to the minimal 35S promoter of *Cauliflower mosaic virus* (mp35S) before the GFP gene. The putative DOF (AAAG) and MYBSt1 (GGATA) core motifs are indicated.

(B) and (C) Relative activities (GFP expression) of the substituted *AtEXP7* promoters in the wild-type (WT) root (B) and in the *rhd6* root with 5 μ M ACC treatment (C). Bars indicate standard errors ($n = 15$ to 32).

(D) Relative activities of the gain-of-function *AtEXP7* promoters in the wild-type root. Bars indicate standard errors ($n = 9$ to 14). E7G4 contains the -134/-46 region (wild-type promoter), and E7G4M6 and E7G4M7 are the same as the E7G4 construct but with E7M6 and E7M7 substitution mutations, respectively.

(E) to (G) Confocal microscopy images of roots harboring the gain-of-function *AtEXP7* promoters mp35S (E), E7G4 (F), and E7G4M6 (G) (a similar expression pattern was observed with E7G4M7).

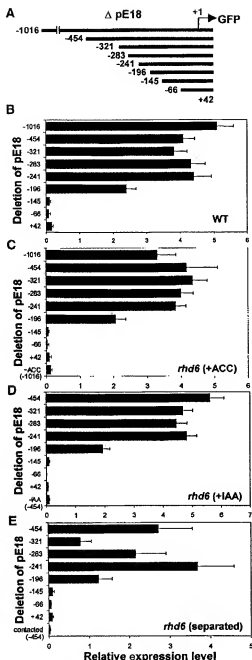


Figure 10. Deletion Analysis of the *AtEXP18* Promoter.

(A) Deletions of the *AtEXP18* promoter ($\Delta pE18$) that are fused to the coding region of GFP. Numbers indicate nucleotide positions relative to the transcription initiation site (+1).

(B) Relative activities (GFP expression) of the truncated *AtEXP18* promoters in the wild-type (WT) root. Bars indicate standard errors.

(C) to (E) Relative activities of the truncated *AtEXP18* promoters in the *rhd6* root. For gene induction, the transformed mutant seedlings

DISCUSSION

Ethylene and Root Hair Development

Recent studies have contributed significantly to our understanding of cell fate determination in the Arabidopsis root epidermis. However, the morphogenetic process of root hair development, which is regulated by hormones and environmental factors, has remained less characterized. In this study, we examined the role of endogenous ethylene and the hierarchical relationship between ethylene, auxin, and an environmental factor (root separation from the agar medium) in root hair initiation. To understand the action of these factors at the gene regulation level, we adopted two expansin genes, *AtEXP7* and *AtEXP18*, whose expression is linked tightly to root hair initiation, as molecular markers.

The involvement of ethylene in root hair formation has been demonstrated in genetic and pharmacological studies. Treatment with the ethylene precursor ACC and mutations of *ctr1* and *eto* induced additional root hairs from the cells in the N position (Dolan et al., 1994; Masucci and Schiefelbein, 1994, 1998; Tanimoto et al., 1995; Cao et al., 1999) (Table 1), and these factors also could restore root hairs in the root hair-defective *rhd6* mutant (Masucci and Schiefelbein, 1995). Although these results clearly show that ethylene is a positive effector of root hair formation, they are indicative of the effect of a constitutive ethylene response and excessive (or exogenous) ethylene but not of the effect of the normal endogenous ethylene level. The mutation of *CTR1* causes constitutive ethylene responses regardless of the absence or presence of ethylene, and the *eto* mutants produce excessive ethylene from 2- to 100-fold (Kieber et al., 1993).

The ethylene biosynthesis inhibitor AVG has been used to show the role of endogenous ethylene, which greatly inhibits root hair formation (Masucci and Schiefelbein, 1994, 1998; Tanimoto et al., 1995) (Table 1). However, AVG likely has toxicity to root hair development, because it completely inhibited root hair formation and the expression of *AtEXP7* and *AtEXP18*, even in the *ctr1* mutant (Figure 3, Table 1).

The role of endogenous ethylene in the wild type can be assessed by the use of mutations that block the ethylene responses. A previous study reported that the dominant ethylene receptor mutant *etr1* maintains normal root hair density (Masucci and Schiefelbein, 1998), thereby raising doubt about the role of endogenous ethylene during the normal (default) process of root hair formation. In Arabidopsis, there are five ethylene receptors whose physiological function, in

were treated with 5 μ M ACC (C) or 30 nM IAA (D) or roots were separated from the agar medium (E) for 1 day before observation. Bars indicate standard errors. In (B) to (E), $n = 25$ to 40.

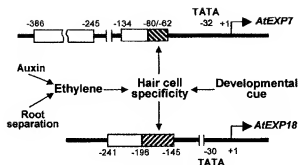


Figure 11. Summary of Promoter Analyses of *AtEXP7* and *AtEXP18*.

The hatched boxes represent elements for hair cell specificity, and the open boxes represent elements that are likely to be relevant to promoter strength. The environmental (root separation) and hormonal signals converge on the elements for hair cell specificity. Numbers indicate nucleotide positions relative to the transcription initiation site (+1). TATA indicates the TATA box.

terms of the triple response, is similar. Dominant mutations in these receptors negatively regulate ethylene responses by constitutively activating CTR1, the negative regulator of downstream ethylene responses. Thus, a dominant mutation in any one of the receptors is able to suppress ethylene responses (Hua and Meyerowitz, 1998). In spite of this genetic principle, inhibition by the dominant mutation shows a dosage-dependent response according to the number of mutant loci and also shows different degrees of phenotypic effect among the five receptors (Hall et al., 1999). Therefore, the contribution of ethylene receptors to root hair formation might depend on receptor species and their temporal/spatial expression pattern.

To determine whether ethylene receptors other than ETR1 are involved in root hair formation, we examined the effect of dominant mutations in all five ethylene receptors. Furthermore, because multiple receptors might be involved in root hair formation, the specific ethylene antagonist 1-MCP was used to simultaneously inhibit ethylene binding by different ethylene receptors. Our results showed that neither the dominant mutations of ethylene receptors nor 1-MCP treatment substantially reduced root hair numbers and the expression of *AtEXP7* and *AtEXP18* (Figures 3 and 7, Table 1), indicating that endogenous ethylene is not required for normal (default) root hair formation in the wild type.

Ethylene, however, is likely to mediate auxin- or root separation-induced root hair formation. Blocking the ethylene perception by 1-MCP almost completely inhibited auxin- or root separation-induced root hair formation and expression of *AtEXP7* and *AtEXP18* (Figures 5 and 6). Auxin and certain biotic/abiotic factors, such as pathogens, wounding, chilling, hypoxia, and water stress, are well-known stimulators of ethylene biosynthesis (McKeon et al., 1995). Localized

water stress could develop in the root when it is separated from the agar medium or exposed to air, a treatment that is known to stimulate root hair elongation (Okada and Shimura, 1994). Therefore, auxin and root separation may induce root hair initiation through an increase in ethylene production, although we do not exclude the possibility that these treatments affect components of ethylene signaling.

A previous study suggested that ethylene and auxin take separate pathways to affect root hair development. Auxin restored root hairs in the AVG-treated root and in the *aux1 etr1* double mutant (Masucci and Schiefelbein, 1996). However, the latter case indicates a complicated aspect of root hair development, because ACC significantly suppressed root hair formation in the double mutant rather than simply having no effect on the restoration of root hairs. A similar perplexing result from the same study is that ACC also inhibited root hair formation considerably in the *rdh6 eth2* double mutant. It appears that excessive ethylene (or its precursor) inhibits the ethylene responses of ethylene-insensitive mutants in certain conditions.

The dominant mutant *axr2* maintains 64% of root hairs compared with the wild type, and ACC or auxin only partially restores the root hair number in the mutant (74 to 81% compared with the wild type) (Masucci and Schiefelbein, 1996). The *axr2* mutant carries the gain-of-function mutation in an Aux/IAA transcriptional repressor (IAA7) so that the mutant molecule is resistant to the auxin-mediated degradation process (Nagpal et al., 2000; Tiwari et al., 2001). The *axr2* plant can be less sensitive to ACC if *AXR2/IAA7* represses expression of the components of ethylene signaling or if the gain-of-function mutant protein finds new targets, such as genes required for the root hair initiation machinery, as a result of its durability time and concentration in the nucleus. The epistatic effect of *axr2* over *tig* or *gl2* (Masucci and Schiefelbein, 1996) could be acquired if the latter case occurs.

In contrast to root hair initiation, root hair elongation is dependent on endogenous ethylene. Blocking ethylene perception by gain-of-function mutations of the ethylene receptors or by 1-MCP markedly inhibited root hair elongation (Table 2). The difference in ethylene action on the initiation and elongation of root hairs leads us to propose that the two responses have different sensitivities to ethylene. Root hair initiation may require a higher ethylene level than does the root hair elongation process. Treatment with ACC, auxin, or other stimuli is required to exceed the ethylene concentration needed to stimulate root hair initiation, whereas the lower endogenous ethylene level is sufficient to regulate root hair elongation. Alternatively, it is conceivable that ethylene biosynthesis increases during root hair elongation. Genetic studies indicate that different sets of gene products are instrumental for the root hair initiation and root hair elongation steps (Parker et al., 2000; Schiefelbein, 2000). This finding implies that the two ethylene-dependent responses in a single root hair cell result from the activation of different genetic pathways by different ethylene levels.

Two Different Pathways Manipulate Root Hair Initiation

RHD6 is likely to be a major regulator in the developmental pathway (through TTG/GL2) for root hair formation. Defects in the negative regulator TTG or GL2 induce root hairs from the cells in the N position as well as in the H position. However, root hair numbers in *ttg* and *gl2* mutants are reduced greatly by the defect in RHD6, indicating that RHD6 is an important downstream regulator of the TTG/GL2 pathway (Masucci and Schiefelbein, 1996). The fact that auxin, ethylene, and root separation can restore root hairs in *rhb6* led us to a scheme, illustrated in Figure 12, whereby the separate environmental/hormonal signaling pathway converges with the normal developmental pathway downstream of RHD6. We show the environmental signal (root separation from the medium) as separate from the auxin pathway because root separation restores normal root hair growth in the *aux1* mutant (Okada and Shimura, 1994).

However, the environmental/hormonal pathway appears to have a differential influence on the two epidermal positions (H and N). This is seen clearly in the *rhb6* background (Table 1), in which 5 μ M ACC stimulated root hair formation in the H position but had negligible effect in the N position. Even higher levels of ACC (50 μ M), as well as the *ctr1* and *eto* mutations, induced only some of the cells in the N position to form root hairs (Dolan et al., 1994; Masucci and Schiefelbein, 1996; Cao et al., 1999) (Table 1). This differential response could result from a lower ethylene (or ACC)

sensitivity of cells in the N position compared with cells in the H position (Dolan, 1997; Cao et al., 1999).

Because the occasional root hairs in *rhb6* emerge in abnormal cell positions, RHD6 was implicated in the control of hair cell polarity (Masucci and Schiefelbein, 1994). Cell specification seems to be normal in the *rhb6* mutant, because the distinctive cytoplasmic characteristics between H- and N-positioned cells are the same as in the wild type; apparently, only the hair-inducing machinery is impaired (Masucci and Schiefelbein, 1996). We found that the *rhb6* mutation inhibited the expression of both *AtEXP7* and *AtEXP18* almost completely (Figures 2K, 3, 8, and 10), suggesting that the molecular function of RHD6 is to regulate gene expression in the root hair cell either as a transcriptional regulator or as its upstream component. RHD6 may regulate the expression of the hair cell genes necessary for hair initiation, such as those involved in cytoskeletal dynamics, localized secretion, wall loosening, and wall synthesis. However, RHD6 probably does not target *AtEXP7* and *AtEXP18* directly, because expansin gene expression in *rhb6* can be restored by hormonal and environmental treatments. Our promoter analyses of the two expansin genes showed that ethylene, auxin, and root separation signals require the same promoter elements that control cell specificity (Figure 11). Thus, we propose that the signals from the developmental and environmental/hormonal pathways are merged at or before the transcription regulators that direct the hair cell specificity of the expansin genes. Identification and characterization of these transcription regulators will be important for understanding the mechanism of pattern formation in the root epidermis.

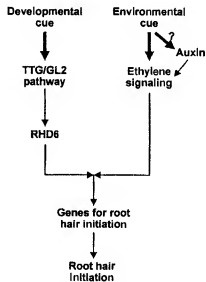


Figure 12. Model illustrating how two separate signaling pathways from Developmental and Environmental cues merge to regulate root hair initiation in *Arabidopsis*.

Arrows designate the information flow.

METHODS

Plant Materials

Arabidopsis thaliana was the model plant in this study. Unless indicated otherwise, the wild type was the Columbia ecotype. The mutant seeds of *ttg-1* (CS89), *gl2-1* (CS65), *eto2* (CS8059), *etr1-1* (CS237), *ers2-1* (CS8854), *ein4* (CS8053), *gtr1-1* (CS8057), and *ein2-1* (CS3071) were obtained from the ABRIC (Columbus, OH). *rhb6* seeds were obtained from J.W. Schiefelbein (University of Michigan, Ann Arbor, MI). Seeds of the gain-of-function mutants *etr2* and *ers1* (Hua et al., 1995) were obtained from J. Hua (Cornell University, Ithaca, NY), and seeds of the loss-of-function mutant *etr1-7* were from E. Schaller (University of New Hampshire, Durham, NH). The seeds were sowed on agar plates including 4.3 g/L Murashige and Skoog (1962) nutrient mix (Sigma), 1% Suc, 0.5 g/L Mes, pH 5.7, with KOH, and 0.8% phytagar. After vernalization for 3 days, the seeds were germinated at 23°C under continuous light. For pharmacological experiments, 3-day-old seedlings were transferred to new plates containing growth regulators or antagonists and incubated for 1 additional day, after which root hairs and reporter gene expression patterns were examined. Transformed plants were selected on hygromycin-containing plates (10 μ g/mL).

RNA Gel Blot Analyses

Total RNA preparation and RNA gel blot analyses were conducted as described previously (Cho and Kende, 1997). Gene-specific probes for *AtEXP7* and *AtEXP18* were generated from 3' untranslated regions of the genes. To confirm equal amounts of RNA loading, the membranes were rehybridized with the Arabidopsis actin2 probe. Transcript levels were quantified from autoradiographs using Adobe Photoshop (Adobe Systems, San Jose, CA) as described previously (Cho and Cosgrove, 2000).

Reporter Gene Constructs

For the reporter gene constructs, the *AtEXP7* promoter region (between -1380 and +48 bp relative to the predicted transcription initiation site) from Arabidopsis BAC F5011 was inserted into HindIII-XbaI sites of the binary vector pGPTV-HYG (Becker et al., 1992), which resulted in the *AtEXP7* promoter::uidA (β -glucuronidase [GUS]) construct. For the *AtEXP7* promoter::green fluorescent protein (GFP) construct, the *uidA* gene was replaced with the gene for GFP. The coding region of GFP was obtained from the pEGFP vector (Clontech, Palo Alto, CA) by PCR using primers 5'-AGTTGGAGC-TCTCAGATCGC-3' (with the SacI site) and 5'-ATCCCGGGTACG-GTCC-3' (with the SmaI site). This fragment of the GFP coding region replaced the *uidA* gene between the SacI and SmaI sites of the *AtEXP7* promoter::GUS construct. For the *AtEXP7* promoter::AtEXP7-GFP construct, in which the *AtEXP7* promoter directs the expression of the *AtEXP7*-GFP fusion protein, the coding region of *AtEXP7* was amplified from the genomic *AtEXP7* done by PCR using primers 5'-CCTAAGAAATCTAGAAAAGAGGCTAGAATG-3' (with the XbaI site) and 5'-AAAAGCCCGGTAAACAGGAATATAGC-3' (with the SmaI site). This fragment was inserted into XbaI-SmaI sites of the *AtEXP7* promoter::GFP construct. All of the constructs were confirmed by DNA sequencing. The constructs were introduced into Arabidopsis plants by *Agrobacterium tumefaciens* strain C58C1 (pMP90) using the vacuum infiltration method (Bechtold and Pelletier, 1998).

Detection of Reporters

GUS staining was performed as described previously (Cho and Cosgrove, 2000). For the detection of GFP, fluorescence from the seedling root was observed with a confocal laser scanning microscope (LSM-410; Carl Zeiss, Jena, Germany). For the cross-sectional view, 1- to 2-mm root sections were made after embedding the root in 1% agarose. To outline the cell boundary in some samples, the root was stained with propidium iodide (10 μ M/L). Green fluorescence was detected by excitation at 488 nm and emission at 543 nm. Red fluorescence from propidium iodide was detected by excitation at 568 nm and emission at 617 nm. Fluorescence images of the separate channels were digitized with LSM software version 3.5 (New Freedom, PA) and merged using Adobe Photoshop. The false red and green colors were adopted for propidium iodide and GFP fluorescence, respectively.

Observation of Root Hair Number and Length

The number of root hairs was determined using a differential interference contrast microscope according to Masucci and Schiefelbein (1996) with some modifications. For each seedling root, 5 consecu-

tive epidermal cells from the same cell file were observed, and a total of 20 cells from two hair cell files and the adjacent two nonhair cell files were counted. Seven to 13 roots (for a total of 140 to 260 cells) per treatment or genotype were scored. Any protrusion was scored as the presence of the root hair, regardless of the length. In the root separated from the agar medium, total root hairs from the separated region were counted. For root separation, the agar medium immediately below the root tip was cut out, and the root was left to grow to the air. Root hair length was measured using a stereomicroscope when the root hair reached the maximum length. Seven root hairs per plant and five plants per genotype or treatment (for a total of 35 root hairs) were scored.

Treatment of 1-Methylcyclopropene

SmartFresh (0.14% 1-methylcyclopropene [1-MCP]) was obtained from H. Warner at Rohm and Haas (Spring House, PA). 1-MCP gas was produced by mixing the powder with water in a tightly sealed container according to the manufacturer's protocol. The gas was administered to the seedlings so that the final concentration was 1 or 10 μ L/L in the container.

Promoter Analyses

The mutated *AtEXP7* promoters with 5' deletions were prepared by PCR using the same reverse primer (5'-GGACCCATTCTAGAC-TCTTT-3', containing the XbaI site) from the 3' end (+48 bp) and the forward primers (containing the HindIII site) from the various 5' ends, as indicated in Figure 8A, with the Arabidopsis BAC F5011 clone as a template. Deletion of the *AtEXP7* promoter was performed similarly by PCR using a reverse primer (5'-TTTACTCTAGATCT-TGAGGGCGCT-3', containing the XbaI site) from the 3' end (+42 bp) and the forward primers (containing the HindIII site) from the 5' ends, as shown in Figure 10A, with the Arabidopsis BAC F16P17 clone as a template.

Substitution mutagenesis of the proximal region (-134 to -70 bp) of the *AtEXP7* promoter, designated E7M1-E7M7 in Figure 9A, was performed using the "megaprimer PCR" method (Barik, 1995). The megaprimers were amplified using the forward primer 5'-TAGTTA-AGCTTTGGAAACGTAA-3' (located at -386 bp and containing the HindIII site) and the mutagenized reverse primers from the regions indicated in Figure 9A. The second PCR was performed with these megaprimers and the same reverse primer that was used for the deletion analysis.

The gain-of-function promoters of *AtEXP7* were made by associating diverse lengths of proximal promoter parts with the minimal 35S promoter of *Caulliflower mosaic virus* (mp35S). The mp35S region (-64 35S promoter; Eyal et al., 1995) was produced by PCR using the forward primer 5'-AAGGGTCTAGACACAAATCCCACTA-3' (containing the XbaI site) and the reverse primer 5'-GACACCCCGGG-ATCCCACTA-3' (containing the SmaI site) from the pBI121 vector (Clontech). This PCR product was inserted between the XbaI and SmaI sites of pGPTV-HYG so that mp35S was followed by the GFP reporter gene. The gain-of-function *AtEXP7* promoters E7G1 to E7G3, as shown in Figure 9A, were prepared by complementing the sense and antisense oligonucleotides, which contained HindIII and XbaI sites at their 5' and 3' flanking regions, respectively. The E7G4 gain-of-function promoter was produced by PCR amplification of the region between -134 and -46 bp. E7G4M6 and E7G4M7 were

made from the same region from which E7G4 was made except that PCR was performed with the E7M6 and E7M7 constructs as templates, respectively.

The truncated or substituted promoter fragments were inserted between the HindIII and XbaI sites of the pGPTV-HYG vector so that the promoters were followed by the GFP reporter gene, and the gain-of-function promoters were inserted between the HindIII and XbaI sites of the pGPTV-HYG vector containing mp35S::GFP. The constructs were introduced into *Arabidopsis* plants (either the wild type or *rhod6*) using *Agrobacterium* as described above. The first generation of transformants (T1, 9 to 62 independent lines per construct) was used to quantify the relative expression levels of GFP in the root. After selection for 5 days on hygromycin-containing plates, the transformants were transferred to new plates without effectors for the wild-type background or with 1-aminocyclopropane-1-carboxylic acid (5 μ M), indole-3-acetic acid (30 nM), or separation of the root from the agar medium for the *rhod6* mutant, and GFP expression was observed 1 day after the treatments.

To evaluate the promoter activity (GFP expression), fluorescence images of roots were taken digitally using a confocal laser scanning microscope. Relative brightness from the digital images was quantified using the histogram function in Adobe Photoshop. For the histogram analysis, a rectangular marquee (4 \times 3 of the root diameter) was located around the root, where GFP fluorescence is maximal, and the mean value was read from the histogram window. The final relative brightness was calculated by subtracting the background values.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The accession numbers for the genes described in this article are AC025416 (*AtEXP7*), AC011000 (*AtEXP18*), and U41998 (*AtACT2*).

ACKNOWLEDGMENTS

We thank Daniel M. Durachko for technical assistance and Cheryl Granger for kind advice with confocal microscopy imaging. We also are grateful to Drs. Jian Hua (Cornell University), John Schiefelbein (University of Michigan), and Eric Schaller (University of New Hampshire) for kindly sending the mutant seeds and to Dr. H. Warner (Rohm and Haas) for the generous gift of 1-MCP. We also thank the reviewers for helpful comments and suggestions on the manuscript. This research was supported by a grant from the National Science Foundation to D.J.C.

Received July 18, 2002; accepted September 15, 2002.

REFERENCES

Abel, F.B. (1985). Ethylene and plant development: An introduction. In *Ethylene and Plant Development*, J.A. Roberts and G.A. Tucker, eds (London: Butterworths), pp. 1–8.

Baranowskij, N., Froberg, C., Prat, S., and Wilmitzer, L. (1994). A novel DNA binding protein with homology to Myb oncoproteins containing only one repeat can function as a transcriptional activator. *EMBO J.* 13, 5283–5292.

Barik, S. (1995). Site-directed mutagenesis in vitro by megaprimer PCR. *Methods Mol. Biol.* 57, 203–215.

Bechtold, N., and Pelletier, G. (1998). In *plant Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration*. In *Arabidopsis Protocols*, J.M. Martinez-Zapater and J. Salinas, eds (Totowa, NJ: Humana Press), pp. 259–266.

Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992). New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* 20, 1195–1197.

Bibikova, T.N., Jacob, T., Dahse, I., and Gilroy, S. (1998). Localized changes in apoplastic and cytoplasmic pH are associated with root hair development in *Arabidopsis thaliana*. *Development* 125, 2925–2934.

Cao, X.F., Linstead, P., Berger, F., Kieber, J., and Dolan, L. (1999). Differential ethylene sensitivity of epidermal cells is involved in the establishment of cell pattern in the *Arabidopsis* root. *Physiol. Plant.* 106, 311–317.

Carey, M., and Smale, S.T. (2000). Transcriptional Regulation in Eukaryotes. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Cho, H.-T. (2001). Modulation of plant organ growth by expansins, the cell wall loosening proteins. *AgBiotechNet* 3, ABN 069 <http://www.agbiotech.net/reviews/available.asp>.

Cho, H.-T., and Cosgrove, D.J. (2000). Altered expression of expansin modulates leaf growth and petiole abscission in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 96, 9783–9788.

Cho, H.-T., and Kende, H. (1997). Expression of expansin genes is correlated with growth in deepwater rice. *Plant Cell* 9, 1661–1671.

Cormack, R.G.H. (1947). A comparative study of developing epidermal cells in white mustard and tomato roots. *Am. J. Bot.* 34, 310–314.

Cosgrove, D.J. (1999). Enzymes and other agents that enhance cell wall extensibility. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 391–417.

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. *Nature* 407, 321–326.

Dolan, L. (1997). The role of ethylene in the development of plant form. *J. Exp. Bot.* 48, 201–210.

Dolan, L., and Costa, S. (2001). Evolution and genetics of root hair stripes in the root epidermis. *J. Exp. Bot.* 52, 413–417.

Dolan, L., Duckett, C.M., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, S., and Roberts, K. (1994). Clonal relationships and cell patterning in the root epidermis of *Arabidopsis*. *Development* 120, 2465–2474.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organization of the *Arabidopsis* root. *Development* 119, 71–84.

Eyal, Y., Curie, C., and McCormick, S. (1995). Pollen specificity elements reside in 30 bp of the proximal promoters of two pollen-expressed genes. *Plant Cell* 7, 373–384.

Foreman, J., and Dolan, L. (2001). Root hairs as a model system for studying plant cell growth. *Ann. Bot.* 88, 1–7.

Fowler, J.E., and Quatrano, R.S. (1997). Plant cell morphogenesis: Plasma membrane interactions with the cytoskeleton and cell wall. *Annu. Rev. Cell Dev. Biol.* 13, 697–743.

Galway, M.E., Masucci, J.D., Lloyd, A.M., Walbot, V., Davis, R.W., and Schiefelbein, J.W. (1994). The *TTG* gene is required to spec-

- ity epidermal cell fate and cell patterning in the *Arabidopsis* root. *Dev. Biol.* 166, 740-754.
- Hall, A.E., Chen, Q.G., Findell, J.L., Schaller, G.E., and Bleecker, A.B. (1999). The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. *Plant Physiol.* 121, 291-300.
- Hall, A.E., Findell, J.L., Schaller, G.E., Sisler, E.C., and Bleecker, A.B. (2000). Ethylene perception by the ERS1 protein in *Arabidopsis*. *Plant Physiol.* 123, 1449-1457.
- Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by *Arabidopsis* ERS gene. *Science* 269, 1712-1714.
- Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* 94, 261-271.
- Jackson, M. (1991). Ethylene in root growth and development. In *The Plant Hormone Ethylene*, A.K. Mattoo and J.C. Suttle, eds (Boca Raton, FL: CRC Press), pp. 159-181.
- Kieber, J.J., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. *Cell* 72, 427-441.
- Lee, M.M., and Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in *Arabidopsis*, is a position-dependent regulator of epidermal cell patterning. *Cell* 99, 473-483.
- Lee, M.M., and Schiefelbein, J. (2002). Cell pattern in the *Arabidopsis* root epidermis determined by lateral inhibition with feedback. *Plant Cell* 14, 611-618.
- Lee, Y., Choi, D., and Kende, H. (2001). Expansins: Ever-expanding numbers and functions. *Curr. Opin. Plant Biol.* 4, 527-532.
- Li, Y., Darley, C.P., Ongaro, V., Fleming, A., Schipper, O., Baldauf, S.L., and McQueen-Mason, S.J. (2002). Plant expansins are a complex multigene family with an ancient evolutionary origin. *Plant Physiol.* 128, 854-864.
- Masucci, J., and Schiefelbein, J.W. (1994). The *rhb6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process. *Plant Physiol.* 106, 1335-1346.
- Masucci, J.D., Rerie, W.G., Foreman, D.R., Zhang, M., Galway, M.E., Marks, M.D., and Schiefelbein, J.W. (1996). The homeobox gene *GLABRA 2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. *Development* 122, 1253-1260.
- Masucci, J.D., and Schiefelbein, J.W. (1998). Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. *Plant Cell* 8, 1505-1517.
- McKeon, T.A., Fernández-Maculé, J.C., and Yang, S.-F. (1995). Biosynthesis and metabolism of ethylene. In *Plant Hormones*, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Press), pp. 118-139.
- McQueen-Mason, S., Durachko, D.M., and Cosgrove, D.J. (1992). Two endogenous proteins that induce cell wall expansion in plants. *Plant Cell* 4, 1425-1433.
- Meijer, E.G.M. (1989). Developmental aspects of ethylene biosynthesis during somatic embryogenesis in tissue cultures of *Medicago sativa*. *J. Exp. Bot.* 40, 479-484.
- Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15, 473-497.
- Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpole, C., Estelle, M., and Reed, J.W. (2000). *AXR2* encodes a member of the AUX/IAA protein family. *Plant Physiol.* 123, 563-573.
- Okada, K., and Shimura, Y. (1994). Modulation of root growth by physical stimuli. In *Arabidopsis*, E.M. Meyerowitz and C.R. Somerville, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 665-684.
- Parker, J.S., Cavell, A.C., Dolan, L., Roberts, K., and Grierson, C.S. (2000). Genetic interactions during root morphogenesis in *Arabidopsis*. *Plant Cell* 12, 1961-1974.
- Peterson, R.L., and Stevens, K.J. (2000). Evidence for the uptake of non-essential ions and essential nutrient ions by root hairs and their effect on root hair development. In *Root Hairs*, R.W. Ridge and A.M.C. Emons, eds (Tokyo: Springer), pp. 179-195.
- Pien, S., Wyrzykowska, J., McQueen-Mason, S., Smart, C., and Fleming, A. (2001). Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proc. Natl. Acad. Sci. USA* 98, 11812-11817.
- Pitts, R.J., Cernac, A., and Estelle, M. (1998). Auxin and ethylene promote root hair elongation in *Arabidopsis*. *Plant J.* 16, 553-560.
- Schiefelbein, J.W. (2000). Constructing a plant cell: The genetic control of root hair development. *Plant Physiol.* 124, 1525-1531.
- Sisler, E.C., Dupille, E., and Serek, M. (1996). Effect of 1-methylcyclopropene and methylenecyclopropane on ethylene binding and ethylene action on cut camations. *Plant Growth Regul.* 18, 79-86.
- Tanimoto, M., Roberts, K., and Dolan, L. (1995). Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. *Plant J.* 8, 943-948.
- Tiwari, S.B., Wang, X.-J., Hagen, G., and Guilfoyle, T.J. (2001). AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell* 13, 2809-2822.
- Wada, T., Tachibana, T., Shimura, Y., and Okada, K. (1997). Epidermal cell differentiation in *Arabidopsis* determined by a Myb homolog, CPC. *Science* 277, 1113-1115.
- Wilson, A.K., Pickett, F.B., Turner, J.C., and Estelle, M. (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. *Mol. Gen. Genet.* 222, 377-383.
- Yanagisawa, S., and Schmidt, R.J. (1999). Diversity and similarity among recognition sequences of Dof transcription factors. *Plant J.* 17, 209-214.

EXHIBIT D

A 20 nucleotide upstream element is essential for the nopaline synthase (*nos*) promoter activity

Younghee Kim, Kimberly Buckley, Michael A. Costa and Gynheung An*
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA (*author for correspondence)

Received 24 June 1993; accepted in revised form 13 October 1993

Key words: auxin, methyl jasmonate, nopaline synthase promoter, regulatory element, salicylic acid, wounding

Abstract

The nopaline synthase (*nos*) promoter is expressed in a wide range of plant cell types and regulated by various developmental and environmental factors. The *nos* upstream control region essential for this regulation was studied by means of synthetic oligomers using transient and stable transformation systems. Insertion of a 20 nucleotide sequence containing two hexamer motifs and a spacer region into deletion mutants lacking the upstream control region was essential for promoter activity. Mutation of one or more nucleotides of either hexamer sequence significantly altered the strength of expression of the *nos* promoter. Point mutations within the spacer region also strongly influenced promoter strength. Insertion of multiple copies of the 20 nucleotide sequence into the nonfunctional deletion mutants proportionally increased the promoter activity. These results suggest that this twenty nucleotide sequence is essential for the *nos* promoter to function. Substitution of the *nos* element with the *ocs* or 35S *as-1* which contain similar hexamer motifs restored not only promoter activity but also responses to wounding, auxin, methyl jasmonate, and salicylic acid.

Introduction

The transferred DNA (T-DNA) genes of *Agrobacterium* tumor-inducing (Ti) plasmid are transcribed in transformed tumor tissues [15]. Because several genes are clustered within a short segment of the T-DNA, the regulatory regions of each gene are relatively small compared to those of plant genes. The flanking regions of T-DNA genes carry typical eukaryotic regulatory sequences such as the TATA box, CAAT box, and polyadenylation signal sequences [7]. It has been previously observed that T-DNA transcripts are

polyadenylated and that transcription is inhibited by α -amanitin [19, 42], suggesting that plant transcription factors recognize the regulatory regions of T-DNA genes and can properly initiate transcription.

The regulatory region of the T-DNA gene octopine synthase (*ocs*) has been extensively studied. It has been previously shown that the upstream elements located between 222 and 177 bp from the *ocs* transcription initiation site are important for activity of the *ocs* promoter [30]. This region contains a 16 bp palindrome which is sufficient for activating a maize *Adhl* promoter in

tobacco cells [17] and which interacts with a nuclear protein factor [18, 36, 40]. A cDNA clone encoding OCSBF-1, which binds specifically to the *ocs*-element sequence, was isolated from maize [35]. This transcription factor also binds to the regulatory elements of cauliflower mosaic virus (CaMV) 35S and nopaline synthase (*nos*) promoters, suggesting the presence of a common regulatory sequence. Analyses of promoters from other T-DNA genes such as gene 5 [27], 780 [10], agropine biosynthase [10], mannopine synthase [14, 31], and isopentenyl transferase [13, 37] showed that an *ocs*-like element is also present in these promoters.

Regulatory regions of *nos*, another T-DNA gene which was thought to be constitutively active in various plant tissues, have been used for construction of plant selectable markers [32]. However, it was later shown that the *nos* promoter activity is organ-specific and developmentally regulated [4]. In seedlings, the lower parts exhibit a higher activity compared to upper parts. In older plants, the promoter activity is very low throughout the entire plant except in roots and certain reproductive organs [4]. Promoter activity is inducible by wounding and is further enhanced by auxin in both vegetative and reproductive organs [3]. We have recently observed that the *nos* promoter is also inducible by methyl jasmonate (MJ) and salicylic acid (SA) which have been proposed as signaling substances for plant defense responses [26].

The *nos* promoter consists of the TATA box, CAAT box, and upstream regulatory region [5, 16, 21]. In transient expression analyses, deletion of the TATA box region resulted in about a 10-fold reduction in promoter activity [5], while deletion of the CAAT box region reduced the promoter activity by about four-fold [16]. In transgenic tobacco plants, the CAAT box also plays an important role since deletion of this region from the *nos* promoter resulted in a reduction of promoter activity in both vegetative and reproductive organs. The 5'-regulatory sequence located upstream of the CAAT box region is essential for the *nos* promoter activity. Deletion analysis of this upstream region showed that the

region located between -130 and -112 is necessary for activity of the promoter. The sequences immediately upstream and downstream of this region positively modulate the promoter activity. It was shown that this upstream region is essential for developmental and environmental regulation of the promoter [3, 21]. DNase I footprint experiments demonstrated that this region interacts with activation sequence factor (ASF)-1, which also interacts with regulatory elements of other plant promoters, such as the 35S promoter, *ocs* promoter, and wheat histone H3 promoter [29]. In the present study, we show that a 20 nucleotide sequence containing two hexamer motifs and a spacer region between them is required for promoter activity and that the hexamer motif-containing element is essential for the responses to wounding, auxin, MJ, and SA.

Materials and methods

Bacterial strains and plant materials

Escherichia coli MC1000 (*ara*, *leu*, *lac*, *gal*, *str*) [11] was used as the recipient for routine cloning experiments. *Agrobacterium tumefaciens* LBA4404 [23] containing the Ach5 chromosomal background and a disarmed helper-Ti plasmid, pAL4404, was used for transformation of cultured tobacco cell line NT-1 or tobacco plants, *Nicotiana tabacum* L. cv. Petit Havana SR1.

Synthetic oligonucleotides

Oligonucleotides were prepared using an Applied Biosystems DNA synthesizer. Both strands were synthesized with GATC at the 5' end of each strand. The oligonucleotides were purified with an OPC cartridge (Applied Biosystems), annealed in TE (10 mM Tris-HCl), 1 mM EDTA, pH 8.0) buffer, and ligated into the *Bam* HI site of the *nos* internal deletion mutant pGA939-126/-112 or pGA939-148/-112, or into the *Bgl* II site of the 5' deletion mutant pGA705. The pGA939 plasmids are high copy number replicons and contain an

internal deletion of the *nos* promoter which is fused to the *cat* reporter gene and the T-DNA gene 6b terminator. A *Bam* HI site is located at the deletion points. The plasmid pGA705 is similar to pGA939 except that it contains the 5' deletion mutant -101 with a *Bgl* II site at the deletion point. Orientation of the insert was determined using the dideoxy nucleotide sequencing kit Sequenase Version 2.0 (United States Biochemicals).

Transient and stable transformation analyses

Transient expression assays were performed with 20 µg of DNA and 10⁶ protoplasts prepared from three-day old suspension cultured cells [16]. The *nos* mutant promoters carrying various synthetic oligomers were subcloned into binary Ti-plasmid vector pGA891 that contains the *cat* reporter and the potato proteinase inhibitor II gene terminator. We have shown previously that this terminator enhanced *cat* gene expression probably by increasing termination efficiency or mRNA stability [6]. *A. tumefaciens* LBA4404 carrying the binary vector was cocultured with actively growing suspension cells [2]. Fifty independently transformed calli were selected on MS agar medium containing 200 µg/ml of kanamycin. These calli were pooled and soluble protein was extracted for chloramphenicol acetyltransferase (CAT) assay. Transgenic tobacco plants were obtained by cocultivation of young leaf segments with *A. tumefaciens* [2]. R1 plants were the first generation obtained by selfing individual transgenic plants. CAT activity was measured [2] using the crude extracts standardized at 2 to 120 µg of total soluble protein. One unit of CAT converts 1 nmol of chloramphenicol to acetylchloramphenicol per minute at 37 °C.

Results

We have previously shown that the 5' deletion mutant -130 of the *nos* promoter retained about one third of the wild-type activity while further deletion to -101 resulted in a total loss of activ-

ity [5]. This result indicates that the region between -130 and -101 contains an essential element(s) for *nos* promoter activity. We also demonstrated that internal deletion mutants within this region are not functional [34] in transient and stable expression analyses. We have studied the regulatory elements of the *nos* promoter using a deletion mutant -126/-112.

The upstream region consists of two hexamer motifs

The regulatory element(s) were studied by inserting a series of synthetic oligomers spanning both strands of the promoter region between -130 and -101. Oligomers were inserted into the *Bam* HI site located at the deletion point of the nonfunctional mutant promoter -126/-112 which was linked to the *cat* reporter gene. Therefore, this generated duplication of certain regions of the promoter. This deletion was selected as a basal molecule for this study because the mutant is nonfunctional. These molecules were transferred to suspension cultured tobacco cells, NT1, using a Ti-plasmid vector via the *Agrobacterium*-mediated stable transformation method. Analyses of the transformants showed that insertion of the 30-mer corresponding to the sequence -130 to -101 (Table 1, 130-101) partially restored the promoter activity. These results further confirm that the sequence between -130 and -101 contains an essential element(s) for *nos* promoter activity. In order to locate the regulatory element(s), a series of synthetic oligomers starting at -130 and terminating at different 3' end points was inserted into the deletion mutant -126/-112. Synthetic oligomers 130-106, 130-109, and 130-112 showed approximately two- to three-fold less activity compared to the 30-mer, indicating that the sequence between -112 and -101 may be involved in enhancing promoter activity in calli. The synthetic oligomer 130-114 insertion resulted in a sharp decrease in activity. Because the major decrease in promoter activity occurred upon omission of the nucleotides -114 and -113, the 3' boundary of a regulatory element is most likely

located in this area. Deletion of nucleotides -114 to -117 (ACGT) from the 3' end, providing the shortest oligomer insert 130-118, completely eliminated promoter activity. This deleted region contains the hexamer nucleotide sequence, ACGTCA, which was previously found to be an important regulatory motif for the *ocs* and 35S promoters [17, 28] and which is present in various T-DNA and plant viral promoters [9]. This hexamer motif appears to be an essential regulatory sequence in the *nos* promoter.

While our study of the *nos* promoter elements was in progress, it was reported that the hexamer motif ACGTCA and its inverted complementary sequence TGACGT are essential for activity of the *ocs* promoter [9, 36]. Similar motifs are also present in the CaMV 35S promoter [8]. Mutations in these motifs inhibited binding of a nuclear transcription factor and significantly affected promoter activity [28, 35]. In the *nos* promoter, this hexamer motif sequence is present between -117 and -112 and another hexamer-like sequence

TGAGCT is located between -131 and -126. The first series of synthetic oligomers tested starts at -130 and therefore is lacking one nucleotide of the hexamer-like sequence. To investigate whether the upstream hexamer-like sequence is important for *nos* promoter activity, a series of oligomers starting at -131 and terminating at different 3' endpoints was synthesized and inserted into the deletion mutant -126/-112. Stable expression analyses (Table I) showed that the 131-112 oligomer containing both hexamer motifs exhibited promoter activity about ten-fold greater than that of the 130-112 oligomer. The 131-114 oligomer, in which two nucleotides of the downstream hexamer motif were deleted, showed reduced promoter strength. However, the effect was not as dramatic as that observed in the first series. Deletion of one more nucleotide from the hexamer motif (oligomer 131-115) resulted in an additional large decrease in promoter strength, although a low level of activity was still detectable. The *nos* promoter became completely inactive when five

Table I. Stable transformation analysis of the *nos* upstream element boundaries inserted into deletion mutant -126/-112:

Oligomer	Sequence ¹	Relative strength ² (mean \pm SE)
130-101	GAGCTAAGCACATACGTCAGAAACCATTAT	25.1 \pm 4.8
130-106	GAGCTAAGCACATACGTCAGAAACC	12.8 \pm 1.3
130-109	GAGCTAAGCACATACGTCAGAA	13.6 \pm 0.6
130-112	GAGCTAAGCACATACGTCA	9.4 \pm 2.5
130-114	GAGCTAAGCACATACGT	0.2 \pm 0.1
130-118	GAGCTAAGCACAT	0
131-112	TGAGCTAAGCACATACGTCA	100.0 \pm 16.1
131-114	TGAGCTAAGCACATACGT	4.6 \pm 0.3
131-115	TGAGCTAAGCACATACG	0.5 \pm 0.2
131-117	TGAGCTAAGCACATA	0
131-120	TGAGCTAAGCAC	0
131-112	TGAGCTAAGCACATACGTCA	100.0 \pm 16.1
130-112	GAGCTAAGCACATACGTCA	9.4 \pm 2.5
127-112	CTAAGCACATACGTCA	1.0 \pm 4.6
125-112	AAGCACATACGTCA	0.6 \pm 0.1
123-112	GCACATACGTCA	0
117-112	ACGTCA	0

¹ Bold type indicates hexamer motifs.

² Data are normalized to the 131-112 oligomer and are averages of two to six independent transformation experiments. The relative strength of 100% for 131-112 oligomer is equivalent to 444 units per gram protein. This activity is ca. 60% of the wild-type *nos* promoter.

(oligomer 131-117) or all (oligomer 131-120) of the nucleotides of the hexamer sequence were deleted. These results indicate that the presence of both hexamer motifs is necessary for a high level of promoter activity and that a partial deletion of the elements decreases but does not abolish promoter activity.

The hexamer-like sequence at the 5' boundary of the *nos* upstream regulatory region matches only four out of six nucleotides of the hexamer sequence TGACGT and this sequence is not found in other T-DNA or viral promoters. To study whether this hexamer-like sequence is important or if another regulatory element is present in this region, we generated a third series of synthetic oligomers differing by deletion of the 5' boundary and tested the effects by means of CAT assay as described above. Deletion of a portion or all of the upstream hexamer sequence significantly reduced promoter activity (Table 1), indicating that this hexamer-like sequence is also essential. However, a low level of promoter activity was still detectable even when the entire hexamer sequence was removed (oligomer 125-112). This residual activity was eliminated by deletion of two or more additional nucleotides in the spacer region which is located between the hexamer motifs (oligomers 123-112 and 117-112). We conclude that the twenty nucleotide sequence between -131 and -112 is essential for efficient

expression of the promoter. This region will be called 'the *nos* element'.

The upstream hexamer-like sequence is a weak regulatory element

The upstream *nos* hexamer motif, TGAGCT, differs from the consensus hexamer sequence, TGACGT, at the fourth and fifth positions, as indicated by the underlined nucleotides. Therefore, we have synthesized a 20 nucleotide *nos* element (128-CG) containing the consensus upstream hexamer sequence motif. These two nucleotide changes resulted in about a two-fold increase in promoter activity (Table 2), suggesting that the native *nos* hexamer does not result in activity as strong as the consensus hexamer sequence.

Comparison of various hexamer sequences shows that the sixth nucleotide is least conserved [9, 28]. For example, the 35S promoter *as-1* element possesses two different hexamer sequences, TGACGT and TGACGC [28]. In order to investigate the significance of this nucleotide, point mutations (oligomers 127-G, 127-C, and 127-A) were generated at this position of the *nos* upstream hexamer sequence. As shown in Table 2, these point mutations reduced the promoter strength by about four- to ten-fold.

Table 2. Stable transformation analysis of point mutations inserted into deletion mutant -126/-112.

Oligomer	Sequence ¹	Relative strength ² (mean \pm SE)
131-112	TGAGCTAAGCACATACGTCA	100.0 \pm 16.1
128-CG	CG	225.7 \pm 23.3
127-G	G	24.2 \pm 0.3
127-C	C	10.3 \pm 5.2
127-A	A	18.9 \pm 11.9
123-T	T	11.0 \pm 3.5
123-C	C	14.3 \pm 2.1
130-112	GAGCTAAGCACATACGTCA	9.4 \pm 2.5
123-ATG	ATG	0
120-TGC	TGC	0
119-TA	TA	3.8 \pm 1.7

¹ Bold type indicates hexamer motifs.

² Data are normalized to 131-112 oligomer and are averages of two to six independent transformation experiments.

The spacer region between two hexamer motifs is essential

The two hexamer motifs are separated by eight nucleotides in the *nos* promoter. Similar spacing is observed in other T-DNA and viral promoters [9]. However, there is much variation in the composition of the spacer sequence. Comparison of several T-DNA promoters containing the hexamer motifs showed that the first three nucleotides, AAG, are the most conserved in the spacer region [9]. To study the significance of this conserved sequence, point mutations were introduced at the third nucleotide in the spacer region (Table 2, oligomers 123-T and 123-C). The G-to-T change reduced the promoter strength by nine-fold and the G-to-C change also significantly reduced the promoter function. It was previously noticed that this spacer region contributes to form part of a potential Z-DNA-forming sequence [16]. Therefore, two mutants (oligomers 123-ATG, 120-TGC) were generated in which three nucleotides were substituted but all the changes were purine to purine or pyrimidine to pyrimidine, thus preserving the structure of the potential Z-DNA-forming sequence. These mutants did not show any activity (Table 2). The third mutant (119-TA) was generated by substituting purine to pyrimidine (A to T) at -119 and pyrimidine to purine (T to A) at -118. These changes destroy the Z-DNA-forming potential of the *nos* upstream region. However, this mutant promoter partially retained the promoter activity. These results agree with previous findings [29] that the Z-DNA-

forming potential alone is insufficient for regulating promoter activity and that the actual sequence at the spacer region is important.

*Other hexamer-containing elements can substitute for the *nos* upstream regulatory region*

Since the *nos* element is significantly homologous to the essential regulatory sequences of the *ocs* and 35S promoters, we have investigated whether these elements can activate the nonfunctional *nos* deletion mutant -126/-112. The *ocs* element is a 16 bp palindromic sequence containing only four nucleotides of the consensus hexamers at either end and these sequences are separated by an eight nucleotide spacer region [17, 40]. We added two nucleotides to each end of the *ocs* element to make a 20-mer which is equivalent to the *nos* element. This oligonucleotide is different from the *nos* element in four positions: two in the upstream hexamer motif and two in the spacer region. The results in Table 3 show that the modified *ocs* element was much stronger than the *nos* element and was similar to the mutant *nos* element (128-CG) which contains the consensus sequences in both hexamer motifs. This mutant *nos* element differs from the *ocs* element by two nucleotides in the spacer region at -121 and -119. Therefore, these changes did not influence promoter activity.

The *ocs* element is a palindrome whereas the *nos* element is not. To study the effects of a palindromic sequence on the *nos* promoter, two oligomers were made. The first oligomer, *nos*-RP,

Table 3. Stable transformation analysis of various hexamer elements inserted into deletion mutant -126/-112.

Oligomer	Sequence ¹	Relative strength ² (mean \pm SE)
<i>nos</i>	TGAGCTAAGCACATACGTCA	100.0 \pm 16.1
128-CG	TGAC <u>CG</u> TAAGCACATACGTCA	225.7 \pm 23.3
<i>nos</i> -RP	TGAC <u>CG</u> TAIGTACATACGTCA	114.8 \pm 30.0
<i>nos</i> -LP	TGAGCTAAGCGCITACGTCA	2.7 \pm 0.7
<i>ocs</i>	TGACGTAAGCGCTTACGTCA	285.5 \pm 90.3
<i>as-1</i>	TGACGTAAG--GGATGACGC	111.0 \pm 15.3
<i>nos/as-1</i>	TGAGCTAAG--GGAACGTCA	2.0 \pm 0.9

¹ Bold type indicates hexamer motifs. The nucleotide sequences which differ from the *nos* wild type element are underlined.

² Data are normalized to 113-112 oligomer and are averages of two to six independent transformation experiments.

which is a palindrome of the right 10 bp of the *nos*, was slightly more active than the wild-type *nos* element (Table 3). However, the palindrome of the left half, *nos*-LP, was weakly active. The *nos*-LP spacer sequence is identical to that of the *ocs* element which is approximately forty-fold stronger. Therefore, the significant reduction in promoter activity in *nos*-LP was due to the two nucleotide substitutions of both hexamers, confirming the importance of the hexamer motifs.

The hexamer motifs of the *as-1* element are separated by six nucleotides whereas the T-DNA hexamers are separated by eight nucleotides. If the optimum distance between the hexamers is eight nucleotides, the *as-1* elements would be predicted to function weakly compared to *ocs* or *nos* elements. Another difference between the *as-1* and *nos* is the direction of the hexamer repeat. The results in Table 3 show that a synthetic 18-mer of the *as-1* element was as active as the *nos* 20-mer. To study the relationship between the direction of hexamer motifs and spacer, a hybrid promoter element (*nos/as-1*) was made by combining the *nos* hexamer motifs and *as-1* spacer sequence. However, the *nos/as-1* element was only weakly active. There are two differences between the *as-1* and *nos/as-1* elements. The first difference is the CG-to-GC change in the upstream hexamer and the second is the orientation of the downstream hexamer. The change in hexamer sequence does not appear to be the main reason for weakening of the promoter since the same change (128-CG to 131-112) affected *nos* promoter strength only

by two-fold (Table 2). Therefore, it appears that inversion of the downstream hexamer motif significantly influenced function of the promoter element. Since the inverted hexamer is the natural orientation of the *nos* and *ocs* elements, the orientation of the hexamer motif alone must not be responsible for the reduction of the promoter strength. It appears that the hexamer motifs and spacer sequence may function together and that spacer separation of these elements affects promoter activity.

Immediate upstream region of the nos element is not essential in activation of synthetic oligomers

We have used the *nos* promoter deletion mutant -126/-112 to study various synthetic oligomers. However, this promoter retains a portion of the upstream hexamer motif. It was also shown earlier that the sequence between -150 and -130 positively modulates the *nos* promoter [5]. To investigate whether the truncated hexamer motif or other elements located at the immediate upstream region of the *nos* element are necessary for promoter activity, the oligomers were tested in the internal deletion mutant -148/-112 (Table 4). Insertion of the *nos* element (oligomer 131-112) into the deletion mutant activated the promoter, indicating that the sequence between -148 and -131 is not essential. A shorter oligomer fragment 131-114 containing a two nucleotide deletion in the downstream hexamer motif resulted in reduced

Table 4. Stable transformation analysis of synthetic oligomers using internal deletion mutant -148/-112.

Oligomer	Sequence ¹	Relative strength ² (mean \pm SE)
<i>nos</i>	TCAGCTAAGCACATACGCTCA	100.0 \pm 14.7
131-114	TCAGCTAAGCACATACGT	9.5 \pm 1.6
128-CG	TGACGTAAGCACATACGCTCA	157.4 \pm 25.9
<i>nos</i> -RP	TGACGTATGTACATACGCTCA	94.2 \pm 17.4
<i>nos</i> -LP	TCAGCTAAGCGCTTAGCTCA	1.5
<i>ocs</i>	TGACGTAAGCGCTTAGCTCA	103.9

¹ Bold type indicates hexamer motifs.

² Data are normalized to *nos* oligomer and are averages of two to six independent transformation experiments except where standard error (\pm) is not shown. The relative strength of 100% for 131-112 oligomer is equivalent to 340 units per gram protein. This activity is ca. 45% of the wild-type *nos* promoter.

promoter activity. Conversion of the upstream hexamer motif to the consensus sequence (oligomer 128-CG) enhanced the promoter strength. The oligomers *nos*-RP, *nos*-LP, and *ocs* were also functional in this deletion mutant at levels similar to those obtained with the deletion mutant -126/-112, showing that the immediate upstream sequence did not directly affect the role of various synthetic oligomers.

We have also tested in transient assays the synthetic oligomers with the 5' deletion mutant -101 which lacks the entire upstream region. The *nos* element was functional in the 5' deletion mutant -101, although at a reduced level of ca. 20% (Fig. 1, sample 1) compared to the wild-type *nos* promoter activity (sample 7). Similar results were obtained with the *ocs* and *as-1* elements (data not shown). This further indicates that the hexamer element alone is sufficient for activation of the minimal *nos* promoter but that the surrounding sequences may be needed for enhancing the promoter activity.

Multiple repeats of nos element enhance the promoter activity

We have shown previously that duplication of the *nos* upstream region enhanced the promoter activity [16]. Therefore, we have investigated whether the enhanced expression was due to the *nos* element. This element was multimerized by self-ligation and inserted at the deletion end point

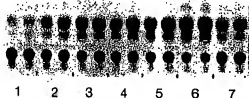


Fig. 1. Transient analysis of multiple hexamer insertion. The twenty nucleotide *nos* element was self ligated and inserted into the minimal *nos* promoter 5'-101. Duplicate results are shown for each sample. Sample 1, single copy; 2, double copy; 3 and 4, four copies in random orientations; 5, six copies; 6, wild-type 35S promoter (between -418 and +1); 7, wild-type *nos* promoter (between -261 and -18). The arrow indicates 3-acetylchloramphenicol.

of the nonfunctional mutant 5'-101. As shown in Fig. 1, an insertion of two copies of the element (sample 2) increased the promoter strength by about five-fold compared to a single copy, returning the amount of expression to a level similar to that of the wild-type *nos* promoter. Insertions of four copies of this sequence (samples 3 and 4) generated about 10-fold stronger promoter activity compared to the single copy. The *nos* promoter carrying six copies of the element (sample 5) exhibited slightly greater promoter strength than the four copy constructs, but still was less than the 35S promoter activity (sample 6).

The nos element is essential for response to wounding, auxin, MJ and SA

It was previously reported that the *nos* promoter is wound inducible and that 2,4-D further enhances the response [3]. We have recently observed that this promoter is also inducible by MJ and SA treatments [26]. Deletion analyses showed that the upstream region, where the *nos* element is present, is essential for these responses. In this study we have further investigated the role of the regulatory upstream region in response to various stimuli using transgenic tobacco plants. The deletion mutant -126/-112, which showed no expression in cultured cells, was also inactive in transgenic plants both before and after treatments by wounding, 2,4-D, MJ or SA (data not shown). This deletion mutant was used for testing insertional activation of promoter activity. The promoter fragments were fused to the *cat* reporter gene and transferred to tobacco plants using a binary Ti-plasmid vector system. For each construct, at least ten transgenic plants were analyzed and all of the experiments were repeated on R1 plants. Although there were variations in the levels of expression among independently transformed plants, the expression pattern and inducibility were similar within each group of transgenic plants carrying the same construct. Figure 2 shows the effects of various stimuli on promoter induction of two randomly selected transgenic plants. Therefore, this data does not necessarily accurately represent the relative strength among

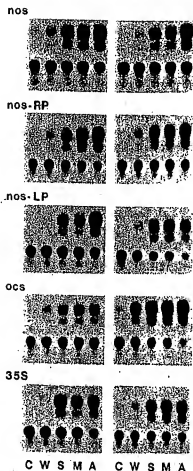


Fig. 2. Wound, SA, MJ, and auxin induction. Fully expanded leaves of transgenic tobacco plants carrying *nos*, *nos-RP*, *nos-LP*, *ocs*, or *as-1* (35S) elements, which were inserted into the internal deletion -126/-112, were sampled before (C) or after 20 h induction by wounding (W), 0.2 mM SA (S), 10 μ M MJ (M), or 0.9 μ M 2,4-D (A). Results from two randomly selected transformed plants are shown for each promoter element. Total soluble protein used for CAT assay was 2 μ g except for *nos-LP* in which 15 μ g was used. The arrow indicates 3-acetylchloramphenicol.

different promoters. Insertion of the *nos* element into the deletion mutant restored wound and auxin inducibility in those plants. The responses to MJ and SA were also recovered by insertion of this oligomer. Shorter fragments (oligomers 131-114, 131-115, 130-112, 127-112) carrying a partial deletion of one hexamer motif also responded to these treatments, although at re-

duced levels (data not shown). Fragments in which either hexamer was completely deleted showed no activity. Oligomers carrying multiple point mutations within the spacer sequences (123-ATG, 120-TGC) also failed to activate the promoter in response to any of these stimuli. However, the oligomers carrying single point mutations (123-T, 123-C) or a double mutation (119-TA) in the spacer region restored the inducibility at reduced levels (data not shown). These experiments confirmed the conclusion from stable calli transformation assays (Table 2) that the *nos* element is needed for the promoter activity and further revealed that responsiveness to the various stimuli is dependent upon the 20 nucleotide element.

Stable transformation experiments with cultured cells demonstrated that palindromes of either the left half (*nos-LP*) or right half (*nos-RP*) of the *nos* element are functional when inserted into the deletion mutant -126/-112. Transgenic plants carrying these oligomers are inducible upon wounding or by chemical treatments (Fig. 2). Transgenic plants carrying the *as-1* or *ocs* element also strongly responded to the wounding or chemical treatments. We have previously demonstrated that the sequence downstream of -101 may not contain an element which is responsive to these stimuli since this minimal promoter fragment, connected to chlorophyll *a/b*-binding protein (*cab*) promoter enhancer region, was not induced by wounding or auxin [3]. This hybrid promoter was also not induced by MJ or SA treatment [26]. To determine whether there is any other element responsible for the induction in the region upstream of -101, the *nos*, *nos-RP*, *nos-LP*, or *ocs* element was connected to the minimal *nos* promoter 5' deletion -101. Transgenic plants carrying these promoters exhibited wound- or chemical-induced expression of the *cat* reporter gene similar to the insertions into -126/-112, although at a reduced level (data not shown).

Discussion

Synthetic oligomers were used to study the *nos* upstream regulatory region. Activity of this pro-

motor is completely destroyed by deletion of the hexamer region [16, 21]. The *nos* upstream region contains an inverted repeat of hexamer motifs of which the most upstream motif differs from the consensus sequence by two nucleotides. A 20 nucleotide sequence, corresponding to the *nos* promoter sequence between -131 and -112, restored promoter activity in nonfunctional deletion mutants. This result agrees with the previous report by Lam *et al.* [29] which showed that insertion of four copies of the synthetic 21-mer between -131 and -111 activated the minimal 35S promoter. It was previously observed that duplication of this region enhanced the *nos* promoter activity [16, 21]. In this study we have demonstrated that multimers of the *nos* element significantly enhanced the promoter function regardless of the orientation, indicating that the upstream element serves as an enhancer. Mutation of one nucleotide in either hexamer motif decreased the strength 10–20-fold. Mutating three or more nucleotides of the hexamer motifs caused a further reduction in promoter activity. Substitution of two consecutive nucleotides of the upstream hexamer motif, resulting in the sequence conforming to the consensus hexamer sequence, enhanced promoter activity by at least two-fold, indicating that this native *nos* upstream sequence is not the optimal hexamer element. The importance of a repeated hexamer element was also observed in the *ocs* promoter [17, 36] and 35S promoter [28]. Single point mutations in the *ocs* hexamer reduced promoter strength by two- to five-fold in a transient assay system. Multiple substitutions of the *ocs* or *as-1* hexamer motifs significantly affected the promoter activity. Our results presented in this study are consistent with these previously reported observations.

We have demonstrated that the eight nucleotide spacer sequence is also important. Point mutations in this region significantly influenced promoter activity as was observed from the hexamer mutations. It was reported earlier that the first three nucleotides (AAG) of this region are more strictly conserved in various spacer regions [9]. Our study showed that a point mutation of the third nucleotide from G to either C or T sig-

nificantly decreased the promoter activity. Three consecutive nucleotide substitutions in the spacer sequence, which allow it to retain the Z-DNA forming potential of the region, completely inactivated the promoter element whereas two nucleotide substitutions that disturbed the purine-pyrimidine rhythm decreased but did not completely destroy the promoter activity. These results suggest that the Z-DNA forming potential alone is not sufficient for the *nos* promoter to function and that the individual nucleotide sequence within the spacer region is important [29].

Both *ocs* and *as-1* restored activity of the non-functional mutant *nos* promoters. Since the *ocs* and *nos* hexamers are present in an inverse orientation whereas the *as-1* motifs occur in a direct repeat, the hexamers must function in both orientations. However, a hybrid element between the *as-1* hexamers and the *nos* spacer sequence was only weakly active. This finding further supports the idea that the hexamer motifs alone are insufficient for activation of the regulatory element and that additional sequences located within the spacer region play an essential complementary role. This result also indicates that the spacer region and hexamer motifs together combine to form one regulatory element. DNA footprinting experiments showed that there are two binding sites within the regulatory elements and that each binding site consists of one hexamer and a portion of the spacer sequence [29, 40]. These results are consistent with our present study, suggesting that a hexamer and a portion of the spacer region together form a subunit. Therefore, it appears that the *nos* element contains two ten-nucleotide subunits, each containing a hexamer motif and a four nucleotide sequence from the spacer region. This hypothesis is further supported by the observation that the palindromes of either the left or right half of the *nos* element are active. The importance of flanking sequences has also been recognized, for example, in association with the G-box motif [41].

We have demonstrated that the *nos* element is sufficient to restore the responsiveness of the minimal *nos* promoter to wounding, auxin, MJ, and SA stimuli. Other hexamer-containing ele-

ments, such as *nos*-RP, *nos*-LP, *ocs* and *as-1*, when substituted for the *nos* element, also resulted in similar responses to these stimuli. Therefore, it is likely that these elements harbor critical sequences which are involved in responses to the stimuli. We have previously reported [3] that the *nos* upstream promoter region, which is essential for the wound and auxin inducibility, contains the conserved sequence GCAN-CATRCRY (where R is purine and Y is pyrimidine) which is also found in other auxin-inducible promoters [1, 12, 22, 33]. Our present study revealed that the conserved sequence (oligomer 123-112) alone is insufficient for the auxin response and that the additional sequence upstream of the region is required. Since auxin response elements have not been characterized in detail from the auxin-inducible genes, it is uncertain whether this conserved sequence is indeed involved in auxin response or if some other sequence such as a hexamer motif-containing element is the primary regulatory element for the response. Because auxin has been shown to regulate a variety of plant genes [20, 25, 39], it is possible that the induction mechanism of one group of genes is different from another group. Therefore, the hexamer element or GCAN-CATRCRY sequence may be present only in certain auxin-inducible promoters.

The observation that the *nos* element can be substituted with other hexamer elements suggests that a common *trans*-acting factor is associated with the control of a variety of hexamer elements. The maize regulatory protein, OCSBF-1, which binds specifically to the *ocs*-element sequence, also binds to the *as-1* and *nos* elements [35]. It was also reported that the tobacco nuclear protein ASF-1, which was identified as a *trans*-acting factor to the *as-1*, also binds to the *nos* hexamer region [29]. We have confirmed and extended these observations by gel retardation and competition studies using the synthetic oligomers and a crude extract prepared from the nuclear fraction of tobacco protoplasts (Y. Kim and G. An, unpublished data).

Several cDNA clones for the transcription factor which specifically interacts with the hexamer

elements have been isolated from various plant species including tobacco [24], maize [35] and wheat [38]. These regulatory factors are members of the bZIP protein family which form homodimers or heterodimers through a leucine zipper dimerization domain. From the suspension cultured tobacco cell NT-1, we have also isolated two cDNA clones which code for similar transcription factors. We are currently investigating how these regulatory factors are involved in expression of the hexamer elements in response to wounding or chemical stimuli.

Acknowledgement

We thank Gregory A. Wilchens for growing the transgenic tobacco plants. This work was supported in part by a grant from the National Science Foundation (DCB-891954).

References

1. Ainley WM, Walker JC, Nagao RT, Key JL: Sequence and characterization of two auxin-regulated genes from soybean. *J Biol Chem* 263: 10658-10666 (1988).
2. An G: Binary Ti vectors for plant transformation and promoter analysis. *Meth Enzymol* 153: 292-305 (1987).
3. An G, Costa M, Ha S-B: Nopaline synthase promoter is wound inducible and auxin inducible. *Plant Cell* 2: 225-233 (1990).
4. An G, Costa M, Mitra A, Ha SB, Marton L: Organ-specific and developmental regulation of nopaline synthase promoter in transgenic tobacco plants. *Plant Physiol* 88: 547-552 (1988).
5. An G, Ebert PR, Yi B-Y, Choi C-H: Both TATA box and upstream regions are required for the nopaline synthase promoter activity in transformed tobacco cells. *Mol Gen Genet* 203: 245-250 (1986).
6. An G, Mitra A, Choi HK, Costa M, An K, Thornburg RW, Ryan CA: Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. *Plant Cell* 1: 115-122 (1989).
7. Barker RF, Idler KB, Thompson DV, Kemp JD: Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi 15955. *Plant Mol Biol* 2: 335-350 (1983).
8. Benfey PN, Chua N-H: The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250: 959-966 (1990).

9. Bouchez D, Tokuhisa JG, Llewellyn DJ, Dennis ES, Ellis JG: The *ocs*-element is a component of the promoters of several T-DNA and plant viral genes. *EMBO J* 8: 4197-4204 (1989).
10. Bruce WB, Bandypadhyay R, Gurley WB: An enhancer-like element present in the promoter of a T-DNA gene from the Ti plasmid of *Agrobacterium tumefaciens*. *Proc Natl Acad Sci USA* 85: 4321-4314 (1988).
11. Casadaban MJ, Cohen SN: Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J Mol Biol* 138: 179-207 (1980).
12. Czarnecka E, Nagao RT, Key JL, Gurley WB: Characterization of Gmhsp26-A, a stress gene encoding a divergent heat shock protein of soybean: Heavy-metal-induced inhibition of intron processing. *Mol Cell Biol* 8: 1113-1122 (1988).
13. de Pater BS, Klinkhamer MP, Amez PA, de Kam RJ, Memelink J, Hoge JHC, Schilperoort RA: Plant expression signals of the *Agrobacterium T-cyt* gene. *Nucl Acids Res* 15: 8267-8281 (1987).
14. Di Rita VI, Gelvin SB: Deletion analysis of the mannopine synthase gene promoter in sunflower crown gall tumors and *Agrobacterium tumefaciens*. *Mol Gen Genet* 207: 233-241 (1987).
15. Drummond MH, Gordon MP, Nester EW, Chilton MD: Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumors. *Nature* 269: 535-536 (1977).
16. Ebert PR, Ha SB, An G: Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays. *Proc Natl Acad Sci USA* 84: 5745-5749 (1987).
17. Ellis JG, Llewellyn DJ, Walker JC, Dennis ES, Peacock WJ: The *ocs* element: a 16 base pair palindrome essential for activity of the octopine synthase enhancer. *EMBO J* 6: 3203-3208 (1987).
18. Fromm H, Katagiri F, Chua N-H: An octopine synthase enhancer directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. *Plant Cell* 1: 977-984 (1989).
19. Gelvin SB, Thomashow MF, McPherson JC, Gordon MP, Nester EW: Size and map positions of several plasmid-DNA-encoded transcripts in octopine-type crown gall tumors. *Proc Natl Acad Sci USA* 79: 76-80 (1982).
20. Guilfoyle TJ: Auxin-regulated gene expression in higher plants. *CRC Crit Rev Plant Sci* 4: 247-276 (1986).
21. Ha S-B, An G: Cis-acting regulatory elements controlling temporal and organ-specific activity of nopaline synthase promoter. *Nucl Acids Res* 17: 215-223 (1989).
22. Hagen G, Martin G, Li MY, Guilfoyle TJ: Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. *Plant Mol Biol* 17: 567-579 (1991).
23. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA: A binary vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303: 179-181 (1983).
24. Katagiri F, Lam E, Chua N-H: Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature* 340: 727-730 (1989).
25. Key JL: Modulation of gene expression by auxin. *BioEssays* 11: 52-58 (1989).
26. Kim SR, Kim Y, An G: Identification of methyl jasmonate and salicylic acid response elements from nopaline synthase (*nos*) promoter. *Plant Physiol* 103: 97-103 (1993).
27. Korber H, Strizhov N, Staiger D, Feldwisch J, Olsson O, Sandberg G, Palme K, Schell J, Koncz C: T-DNA gene 5 of *Agrobacterium* modulates auxin response by auto-regulated synthesis of a growth hormone antagonist in plants. *EMBO J* 10: 3983-3991 (1991).
28. Lam E, Benfey PN, Gilmartin PM, Fang R-X, Chua N-H: Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. *Proc Natl Acad Sci USA* 86: 7890-7894 (1989).
29. Lam E, Katagiri F, Chua N-H: Plant nuclear factor ASF-1 binds to an essential region of the nopaline synthase promoter. *J Biol Chem* 265: 9909-9913 (1990).
30. Leisner SM, Gelvin SB: Multiple domains exist within the upstream activator sequence of the octopine synthase gene. *Plant Cell* 1: 925-936 (1989).
31. Leung J, Fukuda H, Wing D, Schell J, Masterson R: Functional analysis of cis-elements, auxin response and early developmental profiles of the mannopine synthase bidirectional promoter. *Mol Gen Genet* 230: 463-474 (1991).
32. Lichtenstein CP, Fuller SL: Vectors for the genetic engineering of plants. *Genet Engng* 6: 103-183 (1987).
33. McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ: Transcription, organization, and sequence of auxin-regulated gene cluster in soybean. *Plant Cell* 1: 229-239 (1989).
34. Mitra A, An G: Three distinct regulatory elements comprise the upstream promoter region of the nopaline synthase gene. *Mol Gen Genet* 215: 294-299 (1989).
35. Singh K, Dennis ES, Ellis JG, Llewellyn DJ, Tokuhisa JG, Wahleithner JA, Peacock WJ: OCSBF-1, a maize *ocs* enhancer binding factor: Isolation and expression during development. *Plant Cell* 2: 891-903 (1990).
36. Singh K, Tokuhisa JG, Dennis ES, Peacock WJ: Saturation mutagenesis of the octopine synthase enhancer: Correlation of mutant phenotypes with binding of a nuclear protein factor. *Proc Natl Acad Sci USA* 86: 3733-3737 (1989).
37. Strabala TJ, Crowell DN, Amasino RM: Levels and location of expression of the *Agrobacterium tumefaciens* pTIA6 *ipt* gene promoter in transgenic tobacco. *Plant Mol Biol* 21: 1011-1021 (1993).
38. Tabata T, Takase H, Takayama S, Mikami K, Nakatsuka A, Kawata T, Nakayama T, Iwabuchi M: A protein that binds to a cis-acting element of wheat histone genes has a leucine zipper motif. *Science* 245: 965-967 (1989).

39. Theologis A: Rapid gene regulation by auxin. *Annu Rev Plant Physiol* 37: 407-438 (1986).
40. Tokuhashi JG, Singh K, Dennis ES, Peacock J: A DNA-binding protein factor recognizes two binding domains within the octopine synthase enhancer element. *Plant Cell* 2: 215-224 (1990).
41. Williams ME, Foster R, Chua N-H: Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. *Plant Cell* 4: 485-496 (1992).
42. Willmitzer L, Schmalenbach W, Schell J: Transcription of T-DNA in octopine and nopaline crown gall tumors is inhibited by low concentrations of α -amanitin. *Nucl Acids Res* 9: 4801-4812 (1981).

EXHIBIT E

Differential Interactions of Promoter Elements in Stress Responses of the *Arabidopsis Adh* Gene

Rudy Dolferus*, Michel Jacobs, W. James Peacock, and Elizabeth S. Dennis

Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, General Post Office Box 1600, Canberra, ACT 2601, Australia (R.D., W.J.P., E.S.D.); and Vrije Universiteit Brussel, Dienst Plantengenetica, Paardenstraat 65, B-1640 Sint-Genesius-Rode, Belgium (M.J.)

The *Adh* (alcohol dehydrogenase, EC 1.1.1.1) gene from *Arabidopsis thaliana* (L.) Heynh. can be induced by dehydration and cold, as well as by hypoxia. A 1-kb promoter fragment (CADH: -964 to +53) is sufficient to confer the stress induction and tissue-specific developmental expression characteristics of the *Adh* gene to a β -glucuronidase reporter gene. Deletion mapping of the 5' end and site-specific mutagenesis identified four regions of the promoter essential for expression under the three stress conditions. Some sequence elements are important for response to all three stress treatments, whereas others are stress specific. The most critical region essential for expression of the *Arabidopsis Adh* promoter under all three environmental stresses (region IV: -172 to -141) contains sequences homologous to the GT motif (-160 to -152) and the GC motif (-147 to -144) of the maize *Adh1* anaerobic responsive element. Region III (-235 to -172) contains two regions shown by R.J. Ferl and B.H. Laughner (1989) Plant Mol Biol 12: 357–366) to bind regulatory proteins; mutation of the G-box-1 region (5'-CCACGTGG-3', -216 to -209) does not affect expression under uninduced or hypoxic conditions, but significantly reduces induction by cold stress and, to a lesser extent, by dehydration stress. Mutation of the other G-box-like sequence (G-box-2: 5'-CCAAGTGG-3', -193 to -182) does not change hypoxic response and affects cold and dehydration stress only slightly. G-box-2 mutations also promote high levels of expression under uninduced conditions. Deletion of region I (-964 to -510) results in increased expression under uninduced and all stress conditions, suggesting that this region contains a repressor binding site. Region II (-510 to -384) contains a positive regulatory element and is necessary for high expression levels under all treatments.

The expression of the *Arabidopsis Adh* gene (Chang and Meyerowitz, 1986; Dolferus et al., 1990) has many features in common with the extensively studied maize *Adh1* gene (Walker et al., 1987; Olive et al., 1990). The two genes have comparable developmental expression patterns, and both have tissue-specific responses to hypoxic stress. In both maize and *Arabidopsis*, the gene is expressed in seeds, roots, and pollen grains, whereas green aerial plant parts are devoid of detectable levels of ADH activity. In both species, hypoxic induction of the gene occurs in cells of the root system (reviewed by Freeling and Bennett, 1985; Dolferus and Jacobs, 1991; see also Okimoto et al., 1980; Dolferus et al., 1985).

The regions of the *Arabidopsis Adh* promoter responsible

for anaerobic induction have not been defined, but in maize an ARE has been identified (Walker et al., 1987; Olive et al., 1990, 1991a, 1991b). The maize *Adh1* ARE is located between -140 and -99 and consists of two subregions, each containing adjacent GC- and GT-rich domains (Olive et al., 1991a). Mutational analysis showed that in maize both the GC and GT motifs are critical for hypoxic regulation (Olive et al., 1991a, 1991b), and in vivo footprinting revealed that some G residues overlapping with the GT motifs were protected (Ferl and Nick, 1987; Paul and Ferl, 1991). Sequence comparison showed that dicot *Adh* promoters have homology with the ARE, but have only one GT and GC motif in the proximal ARE (Olive et al., 1991b). Although the maize *Adh1* promoter is also inducible by cold stress (Christie et al., 1991), it has not been shown whether the ARE is important for expression under cold-stress conditions.

Ferl and Laughner (1989) have demonstrated in vivo footprinting techniques that three regions in the *Arabidopsis Adh* promoter interact with DNA-binding proteins. Of these footprint areas, the '4C-box' (-147 to -144) has sequence homology to the GC motif of the maize ARE. We refer to this sequence as the *Arabidopsis* GC motif. A GT motif is located just upstream of this GC motif (-160 to -152) in reverse orientation to the GT motifs found in the ARE of maize. This GT motif does not correspond to any of the footprinted areas demonstrated by Ferl and Laughner (1989). We will refer to this GT and GC motif as the presumptive *Arabidopsis* ARE sequence. McKendree and Ferl (1992) suggested that another footprinted area, the G-box element (5'-CCACGTGG-3', position -216 to -209), is functionally important for the *Arabidopsis Adh* promoter under tissue culture conditions. Deletion of this G-box element (5' deletion to position -172) resulted in over 90% reduction in promoter activity, and site-specific mutations in the G-box sequence reduced promoter activity by over 60% in transient expression studies using suspension-cell protoplasts expressing the *Adh* gene constitutively. This system did not allow testing for the involvement of the G-box in hypoxic regulation. The *Arabidopsis* G-box sequence consists of a perfect palindromic sequence that has the sequence requirements for interaction

Abbreviations: ADH, alcohol dehydrogenase; *Adh*, gene encoding ADH; ARE, anaerobic responsive element; GBF, G-box binding factor; GUS, β -glucuronidase; MS, Murashige and Skoog; NOS, nopaline synthase; Nos, gene encoding NOS; NPTII, neomycin phosphotransferase II.

* Corresponding author; fax 61-6-2465000.

with a GBF (Schindler et al., 1992d), and it was shown that a GBF does interact with this G-box (Delisle and Ferl, 1990; McKendree et al., 1990). We will refer to this sequence as the G-box-1 sequence. Another G-box-like sequence is present between positions -191 and -185 (G-box-2: 5'-CCAAGTGG-3'). This sequence is part of a larger footprint area (-195 to -170) and does not have a 5'-ACGT-3' core region, typical of G-box and other sequences binding b-ZIP proteins (Schindler et al., 1992d).

In this paper we present a functional analysis of the *Arabidopsis Adh* promoter in transgenic *Arabidopsis* plants. We have identified the regions critical for hypoxic response. In addition, we show that the promoter is induced by low-temperature stress and dehydration, and we have mapped the sequences essential for these inductions. Our analysis shows that the proximal GC/GT sequence motif, homologous to the maize *Adh1* ARE element, is essential for all three environmental stress responses, and that other upstream elements of the promoter interact differentially with the GC/GT motif in mediating response to the different stresses.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Treatments

Arabidopsis thaliana ecotype C24 seeds were surface sterilized and sown onto MS medium containing 3% Suc and 0.8% agar. After overnight incubation at 4°C to break seed dormancy, the plates were incubated for 1 to 2 weeks at 22°C (16-h/8-h light/dark cycle, 200 $\mu\text{E s}^{-1} \text{cm}^{-2}$) and then transferred to fresh MS plates for another 2 weeks (30–50 plantlets per plate). For treatment with low-oxygen stress, dehydration, and cold stress, 30 to 50 plantlets were harvested under sterile conditions and transferred to Petri dishes containing 15 mL of liquid MS medium. Control plates were incubated for 24 h on a shaking platform in a growth chamber. Low-oxygen treatments were carried out by incubating plantlets in a 99.8% argon atmosphere (anaerobiosis) or by using a 5% O_2 /95% N_2 gas mixture (hypoxic conditions; Howard et al., 1987) for 24 h at 22°C in the dark without shaking. Dehydration treatment was carried out by incubating the plantlets in 15 mL of MS medium, containing 0.6 M mannitol, for 24 h at 22°C on a shaker. This treatment proved to be faster and more reproducible than previously described air-drying methods (e.g. Lång and Palva, 1992) and resulted in visibly heavily dehydrated plants. For cold-stress treatment, the plantlets were incubated on a shaking platform in 15 mL of MS medium at 4 to 5°C for 24 h.

Construction of *Adh* Promoter Deletions and Mutations

Standard methods were used for all recombinant DNA manipulations (Maniatis et al., 1982). The complete *Arabidopsis Adh* gene was isolated as a 3.7-kb *SacI* restriction fragment (Dolferus et al., 1990) containing the *Adh* promoter from -964 with respect to the CAP site and 58 bp of untranslated leader sequence (Fig. 1A). A unique *Bam*HI restriction site was created at +53 using oligonucleotide-directed mutagenesis (in collaboration with Dr. J. Boterman, Plant Genetic Systems, Ghent, Belgium). This promoter fragment is referred to as the "complete" *Adh* promoter (CADH), and was sub-

cloned as a *SacI/Bam*HI fragment in plasmid pGEM3Zf(+). All 5' deletions were generated in this plasmid (pGPADH/Bam) using exonuclease III and S1 nuclease (Erase-a-Base kit, Promega). Plasmid DNA was linearized with *SacI*, to protect the vector, and at an *EcoRV* site at -852 in the *Adh* promoter. After T4 polymerase treatment, *Bgl*II linkers were ligated to the plasmids, cut with *Bgl*II, and self-ligated. Deletion end points were verified by plasmid sequencing, using T7 DNA polymerase and the universal primer.

Site-specific mutagenesis (Kunkel et al., 1987) was used to introduce mutations at four specific regions of the CADH fragment (-964 to +53) of the *Arabidopsis Adh* promoter (Fig. 1B). These regions include three footprint areas determined by Ferl and Laughner (1989), called the G-box-1 (5'-CCACGTGG-3', -218 to -207), the G-box-like sequence G-box-2 (5'-CCAAGTGG, -195 to -170), and the GC motif (-147 to -144), together with the GT motif, homologous to the maize *Adh1* ARE sequence (5'-GGTTT-3', -160 to -152). Mutagenic oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer; their sequences were as follows (bases shown in boldface: are modified base pairs; underlined bases are restriction sites introduced by the mutant oligonucleotides; see Fig. 1E):

Δ G-BOX-1: 5'-CGTTCGCTAGTATTCGGAACATGTTAATTTCTCGTGTATCTTT
 Δ G-BOX-2: 5'-TCTCTCGAACGCTCGGGAACATGTTAGTTGCTAGTATTCGTC
 Δ GT-MOT: 5'-GAATACTAGGGCGTAGGTCATAGAGCGTGTCTCTCTCG
 Δ GC-MOT: 5'-CATCTGTAGAATATTCCTGATTTGGTTTGCC

Correct sequence of the mutants was verified by the presence of the restriction site created by the oligonucleotide (*Afl*III site for G-box-1 and G-box-2; *Xba*I for the GT motif) and by sequencing.

Construction of Chimeric Genes and Binary Vectors for Transformation

Plasmid pHW8 containing the GUS reporter gene, fused to a NOS 3' end, was kindly provided by Dr. J. Boterman. For cloning convenience, this construct was first: subcloned into the polylinker of pGEM3Zf, and the GUS-NOS cassette was isolated from this plasmid as a *Bam*HI/*Hind*III fragment and subcloned into all 5'-deleted and mutagenized *Adh* promoter plasmids. These chimeric genes were then: subcloned as *EcoRI/Hind*III fragments into the polylinker of the binary vector pBIN19 (Bevan, 1984). All binary vectors containing the chimeric gene constructs were mobilized to *Agrobacterium* strain LBA4404 (Ooms et al., 1982) or AGL1 (Lazo et al., 1991), using pRK2013 as a helper strain for triparental matings.

Arabidopsis Transformation Methods

Transgenic *Arabidopsis* plants were obtained using a slightly modified root explant transformation method (Valvekens et al., 1988). Phytigel (Sigma) was included in all media as a solidifying agent (0.3%), and timenin (sodium ticarcillin/potassium clavulanate; Beecham) at a 100 $\mu\text{g/mL}$

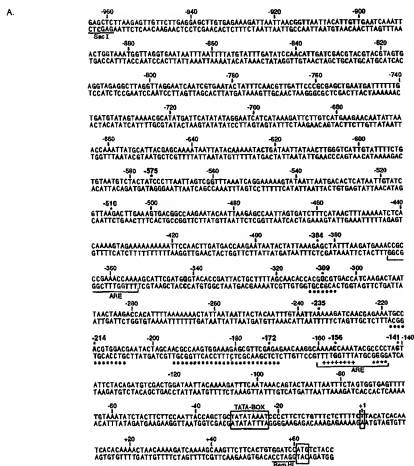
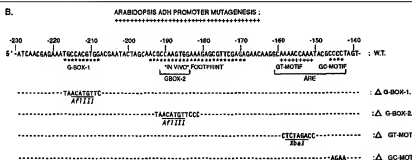


Figure 1. Map of the Arabidopsis Adh promoter. A, The complete Arabidopsis Adh promoter fragment (CADH) used in these studies ranges from position -964 (SacI site) to a BamHI site, introduced at position +53 by site-specific mutagenesis. This fragment was used for all chimeric gene constructs. Base pairs numbered with an asterisk (*) indicate end points of 5' deletions. Asterisks below the sequence indicate regions of the promoter that are protected by proteins, as shown by *in vivo* footprinting (Fehl and Laughner, 1989). Regions homologous to the maize *Adh1* ARE occur at two positions in the Arabidopsis Adh promoter and are indicated by brackets. The first one consists of the GC motif (-147 to -144), together with the sequences between -160 and -152 (GT motif); a second GT/GC-like motif is found between -368 and -354. Other important sequences such as TATA box, transcription start (+1), and translation start of the Adh promoter are indicated. B, Site-specific mutations induced in the CADH (-964 to +53) promoter fragment. Only the region of the CADH promoter between positions -230 and -140 in which the mutations were introduced is shown. Purine-pyrimidine base-pair changes were introduced in the three footprint areas (indicated with asterisks; G-box-1, G-box-2, and GC motif) and the GT motif (+) of the presumptive Arabidopsis ARE sequence.



concentration was used as an antibiotic to eliminate *Agrobacterium*. Shoots (0.5 cm high) were transferred to MS plates containing 50 μ M kanamycin and 100 μ M timentin but not hormones. After 1 to 2 weeks, normal-looking shoots were transferred to Magenta pots (Sigma) containing the same MS medium and F_1 seeds were harvested. NPHT assays were carried out on excised leaves from F_1 plants using the method described by McDonnell et al. (1987). GUS fluorimetric assays and *in vivo* stainings were as described by Jefferson (1987). For *in vivo* GUS and ADH assays, the staining solution was vacuum infiltrated into the plant tissue.

Staining for endogenous ADH activity was carried out at 37°C in the dark in a solution containing 100 mM Tris-HCl, pH 8.0, 10% ethanol, 1 mg/mL nicotinamide adenine dinucleotide, and 200 μ M nitroblue tetrazolium until staining was apparent. Plant material was fixed in 70% ethanol after GUS staining and in a 10% acetic acid solution after ADH staining.

RNA Extractions and Northern Blot Analysis

All RNA analyses were carried out on segregating populations of F_2 or F_3 plants. Total RNA was extracted from

about 1 g of *Arabidopsis* plantlets using a guanidine-HCl extraction method (Logemann et al., 1987). RNA was reprecipitated with 2 M LiCl overnight at 4°C; the concentration was estimated spectrophotometrically, and after a final ethanol precipitation the pellet was dissolved at a 5 µg/µL concentration in diethyl pyrocarbonate-treated water. Equal amounts (25 µg) of total RNA were loaded on 1.1% agarose gels containing 2.2 M formaldehyde in the presence of ethidium bromide (Maniatis et al., 1982). Uniformity of gel loading was judged from the intensity of rRNA bands and other distinct bands in the RNA staining pattern. After electrophoresis the gels were blotted on Hybond-N nylon membranes (Amersham) using 20× SSC as transfer buffer. RNA was immobilized by UV cross-linking. SP6 and T7 RNA polymerase transcription was used to generate [³²P]UTP-labeled probes of the ADH, GUS, NPTII, and *Arabidopsis* ubiquitin (Burke et al., 1988) genes using linearized plasmids as templates. RNA probe hybridizations were carried out in a Hybaid (Hybaid Ltd, Middlesex, UK) oven at 55°C using a hybridization mix containing 50% formamide, 0.25 M NaPO₄ (pH 7.2), 0.25 M NaCl, 1 mM EDTA, and 7% SDS. After hybridization, filters were washed twice at 65°C (30 min each) in PSE (0.25 M NaPO₄, pH 7.2, 2% SDS, 1 mM EDTA), and twice at 65°C (30 min each) in PES (0.04 M NaPO₄, pH 7.2, 1% SDS, 1 mM EDTA), treated with RNase (1 µg/mL in 2× SSC) for 15 min at room temperature, and then washed for 20 min at 50°C in 0.1× SSC/0.1% SDS. Filters were exposed to Fuji RX film and on phosphor screens for analysis and quantification of the hybridization signals with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Analysis of the Transformants and Interpretation of Results

The expression pattern of the transformants was analyzed for four different treatments: uninduced control, hypoxia, dehydration, and cold. Expression was studied at the RNA level, where induction levels from 5- to 50-fold were easily obtained. Where available, at least five independent transformants for one construct were analyzed. Independent transformants of a single construct were analyzed first separately, to verify consistency in the expression pattern of both the chimeric GUS gene and the endogenous *Adh* gene, which was used as a control for the induction treatments. Equal amounts of total RNA from each transformant for every treatment were then mixed and run on RNA gels for northern blot analysis (see Fig. 4A). To verify uniformity of gel loading, RNA gels were stained with ethidium bromide and filters were hybridized with NPTII and ubiquitin probes. To compensate for small differences in gel loading, levels of induction less than twice the control level were not considered significant.

Quantitative variations in expression levels were quite large for each construct. Differences in induction levels between 5- and 70-fold were observed for hypoxically induced samples, and for the same samples the endogenous *Adh* gene showed variations in induction levels between 5- and 20-fold. Various sources of variability can be distinguished. The *Adh* promoter can be switched on partially under supposedly uninduced conditions for various reasons, and the efficiency

of the induction treatment varies between different experiments. For the independent transformants, variation can occur because of position effects, differences in probe strength, and hybridization conditions. Relative GUS expression levels were calculated by dividing GUS mRNA expression levels by their respective endogenous *Adh* gene expression levels. The average as well as the se was determined for each construct and for each treatment (see Table 1 and Fig. 4B). Data for transformants that showed lower than 10-fold inductions of the endogenous *Adh* gene were eliminated, as well as data for transformants that showed clearly aberrant expression behavior (due to position effects) compared to the majority of other transformants.

RESULTS

A 1000-bp Fragment of the *Adh* Promoter Confers Developmental Expression and Tissue-Specific Hypoxic Induction

The -964 to +53 *Arabidopsis Adh* promoter fragment (CADH; Fig. 1) confers the same developmental and differential low-oxygen induction patterns to a GUS reporter gene as are seen with the endogenous *Adh* gene. *Arabidopsis* seeds and plants transformed with the CADH-GUS construct were stained for GUS activity and also for endogenous ADH activity. Both GUS and ADH activity are present in the seed (only in the embryo; Fig. 2A). In germinating seeds and in young seedlings, the *Adh* promoter is functional in cotyledons, the hypocotyl section, and the root tips and stele of the primary root (Fig. 2, B and C). After germination of the seed, *Adh*-driven GUS expression gradually declines in the cotyledon and hypocotyl region, but remains in the root tip and around the vascular bundles of the root. The first leaves show no GUS activity, nor do subsequent rosette leaves (Fig. 2, D and E).

In mature plants, *Adh* promoter-driven GUS activity and expression of the endogenous *Adh* gene are found only in the roots (root cap and root tip, cell elongation zone, and around the vascular bundles; Fig. 2H). The same pattern is observed in lateral roots (Fig. 2I). Under normal conditions, no activity is found in any of the green plant parts (Fig. 2E), except in the apical meristem region (Fig. 2F). Closer analysis shows that GUS activity is present in the base of the rosette leaves and the axial meristems (Fig. 2G). A low activity is observed around the vascular bundles of the stem. GUS expression as well as ADH activity is found in pollen (Fig. 2J). Both CADH-promoter-driven GUS expression and 35S-driven GUS proved to be low in *Arabidopsis* pollen, but with this exception the expression pattern of the *Arabidopsis Adh* gene parallels the developmental expression of the maize *Adh1* gene.

Anaerobiosis induces many higher plant *Adh* genes (Freeling, 1973; Dolferus and Jacobs, 1991; Olive et al., 1991b). The *Arabidopsis Adh* gene was shown to be inducible under anaerobic conditions at both protein (Dolferus et al., 1985) and RNA levels (Dolferus and Jacobs, 1991). In transgenic *Arabidopsis* plants, both the CADH-GUS chimeric gene and the endogenous *Adh* gene are strongly induced by anaerobic conditions (Fig. 3). Maximal induction levels were reached

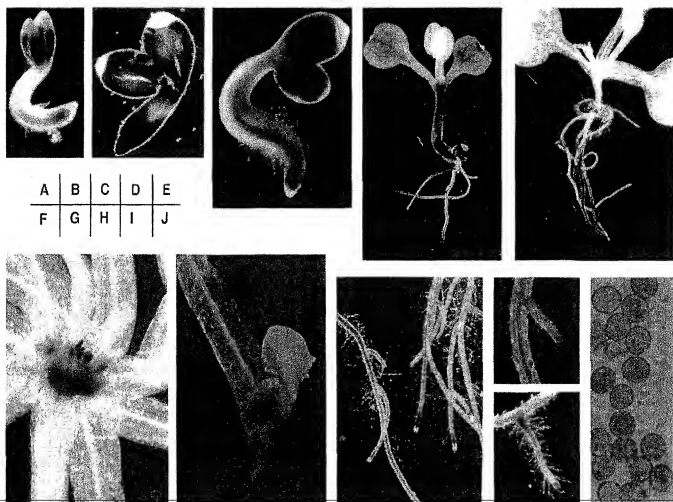


Figure 2. Developmental and tissue-specific expression pattern of *Arabidopsis* plants transformed with an *Adh*-promoter-driven GUS reporter gene (CADH-GUS construct). Expression is found in germinating seeds (A, GUS staining; B, ADH staining), in 4-d-old seedlings (C). In 2-week-old plantlets, activity is present in cotyledons, the hypocotyl, and the root (root tip and along the vascular bundles), but not in the first leaves (D). In mature plants, activity is found in the roots but is absent from the green plant parts (E). However, GUS activity can be demonstrated in the meristematic regions (F; top view of rosettes from 4-week-old plants) and in the base of the leaves (G). Roots of 4-week-old plants show GUS activity in the root tip and along the vascular bundles, in root primordia, and in the lateral roots (H and I). Pollen grains (J) also stain for GUS activity. Essentially the same staining patterns are found as in C to J when ADH activity staining was used (data not shown).

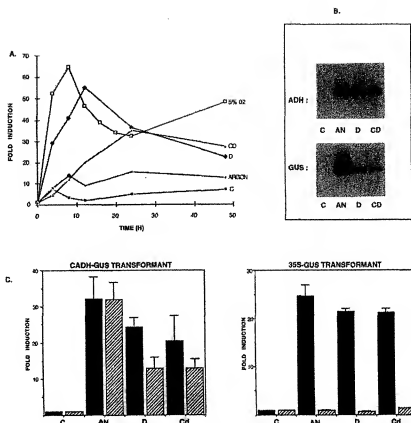
after 4 to 8 h and stayed high for up to 48 h of treatment (Fig. 3A). Although both anoxic (argon) and hypoxic (5% O₂) conditions were shown to have comparable rates of induction, hypoxic conditions gave rise to 4 to 5 times higher expression levels of the endogenous *Arabidopsis Adh* gene (Fig. 3A). Therefore, we used hypoxic or low oxygen (5% O₂) conditions for all further experiments. Staining for ADH and GUS activity shows that expression in plants is restricted mainly to the roots, and the tissue-specific expression is comparable to that in maize. *Adh* mRNA is induced 10- to 50-fold, and the chimeric GUS gene shows equivalent induction (Fig. 3, B and C). Transgenic *Arabidopsis* plantlets, transformed with a 35S-GUS construct, show no increase in expression levels as a consequence of the hypoxic treatment

(Fig. 3C). In conclusion, we find that the CADH promoter fragment contains all the sequence information for tissue specificity and expression under hypoxic conditions.

Regions of the CADH Promoter Fragment Critical for Low-Oxygen Stress Induction

We have analyzed the effect of ten 5' deletions and four substitution mutants of the *Adh* promoter (Fig. 1, A and B) on the expression of the GUS reporter gene in transgenic *Arabidopsis* plants. Deletions in the 5' region removing the sequences to position -510 (region I) cause a quantitative increase in expression under both uninduced and hypoxic conditions (Fig. 4, A and B; Table I). The increase is not

Figure 3. Inducibility of the *Arabidopsis Adh* promoter by environmental stresses, studied at the mRNA level by northern blot hybridization. A, Induction of *Adh* mRNA in unstressed plants (C), anaerobically stressed plants (ARGON), hypoxically-treated plants (5% O₂), dehydration-treated plants (D), and plants treated under low-temperature conditions (CD). B, Northern blot hybridizations, carried out on total RNA isolated from 4-week-old CADH-GUS transformants, show that *Adh* mRNAs as well as GUS mRNAs are strongly induced in plants treated by hypoxia (AN), in plants treated by dehydration stress (D), and in cold-stressed plants (CD). C, Bar diagrams showing -fold inductions for each sample compared to the uninduced control samples: solid black bars, ADH mRNA induction levels; hatched bars, GUS mRNA induction levels (C = 1). Expression of the GUS gene driven by the 35S promoter is not significantly affected by these stress treatments. SE bars are shown for the stressed samples.



significant when the 5' region is deleted to position -575, but a more significant increase under both unstressed and hypoxically induced conditions is seen when the region between -575 and -510 is deleted. A repressor protein(s) may possibly interact with the region between -575 and -510. Deletion of sequences between -510 and -384 (region II) results in a strong decrease (10- to 20-fold) in the hypoxic response, and uninduced expression levels decreased about 4-fold. This indicates that a positive regulatory element is present between positions -510 and -384. McKendree and Ferl (1992) have shown that deletion to position -384 did not reduce GUS expression in suspension-cell protoplasts. The *Arabidopsis Adh* gene is expressed at very high levels in both callus and suspension cultures (Dolferus et al., 1985), and it is not clear what triggers this high expression level. Deletion of the sequences between -384 and -309 removes an ARE-like element between -368 and -354 (Fig. 1A); our data suggest that this element is not critical for *Adh* gene expression under hypoxic conditions. In deletions from -309 onward, uninduced and hypoxically induced expression levels of the *Adh* promoter remain low and gradually decline further. Deletions -172, -156, and -141 show barely detectable hybridization signals under both uninduced and hypoxically induced conditions.

The four substitution mutants (Fig. 1B) defined two other regions of importance. Region III (-235 to -172) contains two regions that are footprinted *in vivo* (Ferl and Laughner, 1989) and include the G-box-1 and G-box-like (G-box-2) sequences. Mutations in both G-box-1 and G-box-2 result in

slightly increased expression under both uninduced and hypoxic conditions compared to the reference CADH-GUS construct (Fig. 4, A and B; Table I). Proteins interact with both G-box sequences in both green plant parts and suspension cells (Ferl and Laughner, 1989), and no changes were seen in the footprint pattern following hypoxic induction. Our data suggest that the G-box-1 and G-box-2 sequences do not play a key role in hypoxic regulation of *Adh* gene expression. Both G-box mutants show comparable behavior under uninduced and hypoxic conditions, but mutations in the G-box-like sequence G-box-2 may cause a more significant increase in expression under both conditions. This could indicate that the two sequences have different functions in the promoter, consistent with the fact that the G-box-2 sequence is not likely to be a binding site for a Leu zipper type of transcription factor (Schindler et al., 1992d).

Mutation analysis defined a second important region located between positions -172 and -141 (region IV). This region shows homology to the GT and GC motifs of the maize ARE (Olive et al., 1990, 1991a, 1991b). Mutation of either the GT or the mGC motif, as well as deletions -156 and -141, which removed these sequences, strongly reduced uninduced expression levels and caused a drastic reduction of expression under hypoxic conditions (Fig. 4). Mutagenesis experiments have shown that both motifs are required for low-oxygen inducibility of the maize *Adh1* promoter (Olive et al., 1990, 1991a, 1991b), and our results show that both the GT and GC motif are critical in the *Arabidopsis Adh*

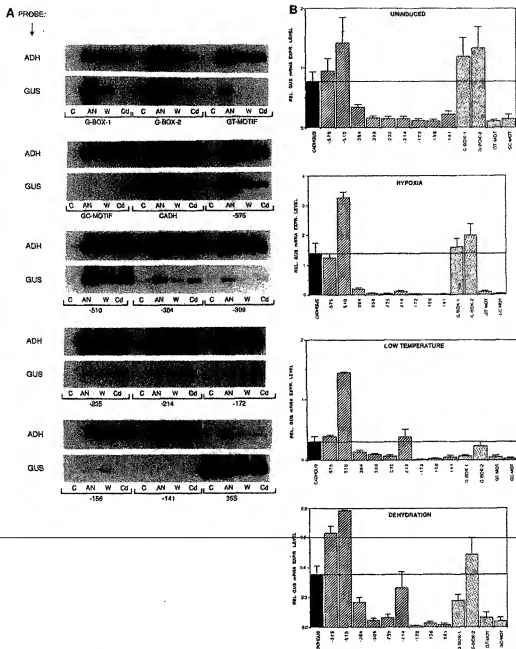


Figure 4. Overview of effects of ten 5'-deletion and four substitution mutants of the *Arabidopsis* *Adh* promoter on mRNA expression patterns under uninduced control (C), low-oxygen (AN), dehydration (W), and cold (Cd) stress conditions. A, Twenty-five micrograms of a total RNA mixture extracted from independent transformants (total plants, 4 weeks old) from each construct (Table I), and only 5 μ g for the 35S-GUS transformant, were loaded on RNA gels. Northern blot filters were probed with GUS and ADH transcription probes as indicated. Equal amounts (cpm) of both GUS and ADH probe were used for hybridization, and exposure length was the same for all samples (2 d). Filters were probed with NPTII and the *Arabidopsis* ubiquitin genes to verify uniformity of gel loading, but the data were not corrected for variation in signal strength observed for these probes, because both the NPTII gene (under the control of the *Nos* promoter) and the endogenous ubiquitin genes were shown to be influenced slightly but consistently by the stress treatments. The NPTII gene was slightly induced by hypoxia, dehydration, and cold stress (on average up to 2-fold; data not shown); the ubiquitin genes were induced by hypoxia and dehydration (up to 1.8-fold on average) and repressed under low-temperature conditions (0.5-fold). Judging from the ethidium bromide staining pattern, gel loading was very uniform and did not correlate with the variations in signal strength observed for the NPTII and ubiquitin probes. B, Bar diagrams showing the changes in average relative expression levels of GUS mRNA for all the constructs under uninduced, low-oxygen, cold, and dehydration stress conditions. Relative GUS mRNA expression levels are determined by dividing the average GUS expression levels by the corresponding average ADH mRNA expression level, as bars are indicated for each sample. The level of expression of the CADH-GUS construct (black bars = 100%) is indicated by a continuous horizontal line for each treatment. Hatched bars show results for 5' deletions; stippled bars indicate expression data for promoter mutants.

Table 1. Summary of results obtained from analysis of 5'-deletion and substitution mutants of the *Arabidopsis Adh* promoter using transgenic plants

Where possible, at least five independent transformants were analyzed per construct. Transformants having abnormal expression patterns compared to the majority of transformants of the same construct, and with low induction levels of the endogenous *Adh* gene, were eliminated (see "Materials and Methods"). For each transformant analyzed, GUS expression levels were divided by the corresponding endogenous *Adh* mRNA expression levels, and these relative GUS expression levels were averaged for each construct and each treatment. The number of independent transformants studied per construct, as well as the averaged relative GUS mRNA expression levels (see values in parentheses) are indicated. Percentages shown below these numbers indicate relative GUS expression levels, compared to the average of the CADH-GUS construct for the same treatment.

Construct	No. Transformants Analyzed	Relative GUS mRNA Expression Levels			
		Uninduced	Low Oxygen	Dehydration	Cold
CADH-GUS (-964)	4	0.764 (0.168) 100%	1.368 (0.354) 100%	0.352 (0.062) 100%	0.302 (0.023) 100%
CADH-GUS ΔG-BOX-1	7	1.186 (0.320) 155.2%	1.582 (0.288) 115.5%	0.175 (0.044) 49.7%	0.061 (0.022) 20.2%
CADH-GUS ΔG-BOX-2	7	1.332 (0.358) 174.3%	1.981 (0.379) 144.8%	0.486 (0.111) 138.1%	0.230 (0.067) 76.2%
CADH-GUS ΔGT-MOT.	6	0.105 (0.026) 13.7%	0.097 (0.031) 7.1%	0.064 (0.035) 18.2%	0.050 (0.026) 16.6%
CADH-GUS ΔCC-MOT.	7	0.147 (0.066) 19.2%	0.030 (0.019) 2.2%	0.039 (0.024) 11.1%	0.027 (0.012) 8.5%
ADH-GUS Δ-575	3	0.941 (0.210) 123.2%	1.239 (0.131) 90.6%	0.629 (0.050) 178.7%	0.389 (0.019) 128.6%
ADH-GUS Δ-510	6	1.415 (0.430) 185.2%	3.263 (0.191) 238.5%	0.785 (0.005) 223.0%	1.445 (0.015) 478.5%
ADH-GUS Δ-384	6	0.340 (0.044) 44.5%	0.191 (0.050) 14.0%	0.167 (0.031) 47.4%	0.138 (0.027) 45.7%
ADH-GUS Δ-309	3	0.161 (0.033) 21.1%	0.052 (0.026) 3.8%	0.044 (0.016) 12.5%	0.091 (0.021) 30.1%
ADH-GUS Δ-235	5	0.142 (0.042) 18.6%	0.040 (0.014) 2.9%	0.063 (0.025) 17.9%	0.065 (0.023) 21.5%
ADH-GUS Δ-214	5	0.144 (0.044) 18.98%	0.111 (0.039) 8.1%	0.261 (0.109) 74.1%	0.382 (0.123) 126.5%
ADH-GUS Δ-172	5	0.111 (0.036) 14.5%	0.024 (0.005) 1.8%	0.011 (0.003) 3.1%	0.009 (0.004) 3.0%
ADH-GUS Δ-156	5	0.103 (0.032) 13.5%	0.011 (0.003) 0.8%	0.024 (0.015) 6.8%	0.025 (0.005) 8.3%
ADH-GUS Δ-141	4	0.216 (0.060) 28.3%	0.017 (0.009) 1.2%	0.017 (0.009) 4.8%	0.046 (0.032) 15.2%

promoter, suggesting that the GT and GC motifs form the *Arabidopsis* equivalent of the maize ARE.

Induction of the *Arabidopsis Adh* Promoter by Cold Stress

Figure 3A shows that the *Arabidopsis Adh* gene is strongly induced by cold stress. The endogenous *Adh* gene is induced 10- to 30-fold at the RNA level and the chimeric gene is induced 5- to 20-fold (Fig. 3, B and C). Our low-temperature induction treatment also induced an *Arabidopsis* cDNA done very similar to the *Arabidopsis* cold-regulated *Cor47* cDNA (data not shown; Gilmour et al., 1992). Cold induction of the *Adh* gene occurs more slowly than hypoxic induction, reaching maximal levels after 24 h (Fig. 3A). The control 35S-GUS construct is not affected by low temperatures (Fig. 3C). These data suggest that the CADH promoter fragment contains sequence information sufficient to confer cold-inducible expression, in addition to the information needed for hypoxic responsiveness. The response of the endogenous *Adh* gene to cold stress was frequently lower than the response to hypoxia,

and this difference was more pronounced with the chimeric gene (Fig. 3, B and C). This could mean that the -964 CADH promoter fragment lacks an upstream activator element for cold response or that an upstream negative element for the hypoxic response is missing. The fact that the CADH-GUS construct is relatively strongly induced by hypoxia, compared to the endogenous *Adh* gene, supports the latter possibility. Alternatively, differences in message stability and the presence of regulatory elements in the coding region of the *Adh* gene, which are absent in the chimeric GUS gene, cannot be excluded.

Cold stress has been associated with increased levels of the phytohormone ABA in plants (Guy, 1990; Hetherington and Quatrano, 1991; Jackson, 1991), but there is no direct evidence that hypoxic stress is correlated with ABA content in plant tissue. Maize plantlets pretreated with ABA were shown to be better able to withstand hypoxic stress, and this increased tolerance was correlated with an increase of ADH activity, but ABA has not been shown to induce maize ADH activity directly (Hwang and VanToai, 1991).

These data pose the question whether induction by low temperature and ABA use the same signal-transduction pathway and involve the same *cis*-acting regulatory sequences. The regions of the maize *Adh1* promoter responsible for induction of gene expression by low-temperature conditions have not been mapped. In *Arabidopsis*, 5' deletions removing the sequences between positions -575 and -510 in region I, which enhance the low-oxygen response, also enhance the low-temperature response of the *Adh* promoter (Fig. 4, A and B; Table I). The results are similar to those seen under uninduced and hypoxic conditions and again suggest the presence of a repressor element between positions -575 and -510. Further deletion of sequences to position -384 (region II) causes a strong reduction in signal strength (Fig. 4, A and B), indicating that a positive regulatory element is present in this region. From position -384 to position -235, the level of cold-stress response remains nearly constant and is stronger than the level of hypoxic expression. With -214 deletions, which keep the core region of the G-box-1 intact, low-temperature expression levels are nearly restored to the CADH-GUS levels. This deletion differentiates the hypoxic and cold-stress inductions, because hypoxic inducibility is not affected in a similar way by this deletion (Table I). Deletions in region IV (-172, -156, and -141) again result in nearly undetectable expression.

Another difference between the two stress responses is found in region III, where mutations of G-box-1 do not affect hypoxic inducibility but do affect cold-stress response (Fig. 4; Table I). In G-box-1 mutants expression is decreased to about 20% of the CADH-GUS construct. In contrast, uninduced and hypoxically induced expression increase for the G-box-1 mutants compared to the CADH-GUS construct. The increase of expression in deletion -214, which leaves the 5'-ACGT-3' core of the G-box-1 sequence intact, and the G-box-1 mutants under both uninduced and hypoxically induced conditions, suggests that the GBF has repressor activities. The low level of cold-induced expression in G-box-1 mutants suggests that the GBF plays an important role in activation of *Adh* expression under cold-stress conditions.

Using transient assays in suspension cell protoplasts, McKendree and Ferl (1992) have shown that a G-box-1 mutant has significantly reduced constitutive expression levels. Our data confirm that the G-box-1 mutants have a lower level of induction by cold but show that they are unaffected in their hypoxic response. We are currently investigating whether induction by ABA is also mediated through this G-box-1 sequence, as is the case for the wheat *Em* gene (Guilting et al., 1990).

Positions -192 to -185 contain a G-box-like sequence (G-box-2: 5'-CCAAGTGG-3'), which does not share the common 5'-ACGT-3' core motif recognized in most b-ZIP binding proteins (Weisshaar et al., 1991; Schindler et al., 1992b, 1992c). The G-box-2 region we have chosen as a target for mutagenesis is part of a larger *in vivo* footprint area (see Fig. 1B). Mutations of G-box-2 resulted in lower cold-induction levels. But reduction in low-temperature response was not as strong as for the G-box-1 mutants, and the signal was hardly discernible above the strong uninduced expression levels (Fig. 4A; Table I).

Mutations of either the GT or GC motif in region IV cause

a drastic decrease in cold-stress response (Fig. 4). These sequences were also critical for expression under uninduced and hypoxic conditions. These data suggest that the ARE, sequence could be more than just an ARE, and that the GT motif together with the GC motif plays a more general role. Data obtained in maize show that a protein interacting with the GC motif of the ARE of the maize *Adh1* gene shares binding site specificity with the human general transcription factor Sp1 (Jones and Tjian, 1985; Olive et al., 1991a). The observation that both hypoxic and cold-stress responses are reduced when the *Arabidopsis* GC motif is mutated suggests that this GC motif may also be a binding site for a general transcription factor.

Induction of the *Adh* Promoter by Dehydration Stress

The suggestion that cold response is related to ABA levels led us to investigate whether other environmental stresses that are correlated with ABA levels, such as dehydration stress (Guy, 1990; Hetherington and Quatrano, 1991; Jackson, 1991), also induce the *Arabidopsis Adh* promoter. We found that the gene is strongly induced when plants are dehydrated in a 0.6 M mannitol solution (Fig. 3). This treatment also strongly induces an *Arabidopsis* cDNA clone with homology to the ABA- and dehydration stress-inducible *Arabidopsis Rab18* gene (Lång and Palva, 1992; data not shown). Maximal induction levels of *Adh* were obtained after 12 h of induction treatment (Fig. 3A). The endogenous *Adh* gene was induced 10- to 30-fold and the chimeric CADH-GUS gene was induced 5- to 20-fold, an induction response comparable to the levels obtained for cold stress (Fig. 3, B and C). Dehydration stress did not influence expression of the control 35S-GUS construct (Fig. 3C). Like cold-stress induction, the dehydration responses of the endogenous *Adh* gene and the GUS-chimeric gene were somewhat lower than the hypoxic response (Fig. 3, B and C).

The inducibility of the *Arabidopsis Adh* gene by both low temperature and by dehydration raises the question whether these stimuli act through the same *cis*-acting promoter elements and share the same signal-transduction pathway involving ABA. As was the case for the uninduced, hypoxic, and cold-stress responses, deletion to position -510 (region I) causes an increase in expression levels under dehydration stress conditions, consistent with the idea that a repressor might interact with this region of the *Adh* promoter (Fig. 4, A and B; Table I). Deletion of sequences between positions -510 and -384 (region II) reduced the dehydration response as it did the low-temperature response and the hypoxic response. This region might bind a general transcription factor that enhances the levels of the stress responses. But region II might also contain additional sequences specific for hypoxic inducibility. Response to dehydration stress remains low for deletions between -384 and -235 (Fig. 4). Deletion -214 in the G-box-1 sequence shows increased expression under dehydration, as was the case for cold. Response to dehydration is undetectable in deletions from position -172 onward.

Mutations in G-box-1 decrease induction by dehydration to about 50% of the CADH-GUS levels (Table I), but not to the same extent as for low temperature. Dehydration stress

induction is not affected significantly in the G-box-2 mutants. This result indicates again that the two G-box sequences may have different functions in the *Adh* promoter. Mutations of the GT and GC motifs cause a drastic reduction in dehydration stress response, as was the case for all other treatments. These data suggest that the *Arabidopsis* ARE sequence could interact with a general transcription factor and that the region is critical for the response of the *Adh* promoter to a variety of different environmental stresses.

Tissue-Specific Expression of the *Adh* Promoter

The three stress responses occur in the same tissues of the plant. Hypoxic response occurs in the roots and is not present

in the leaves, and the same pattern is found in plants stressed by dehydration and cold (Fig. 5, A–D). GUS activity increases over the entire length of the root, especially around the stele, and is most pronounced in the younger roots and lateral roots (Fig. 2, H and I). In the root tip, the expression pattern seen in uninduced roots differed from roots induced by any of the three stresses (Fig. 5E [1–4]). Typically, uninduced roots (Fig. 5E [1 and 3]) show expression in the root tip but less in the elongation zone. The stress-induced GUS expression is strongly increased mainly in the cell elongation zone and along the vascular tissue of the root (Fig. 5E [2 and 4]). Although *Adh* gene expression is root specific, expression can be found occasionally at low levels in leaves. This occurs in wounded leaves (Fig. 5D) and inconsistently in leaves of

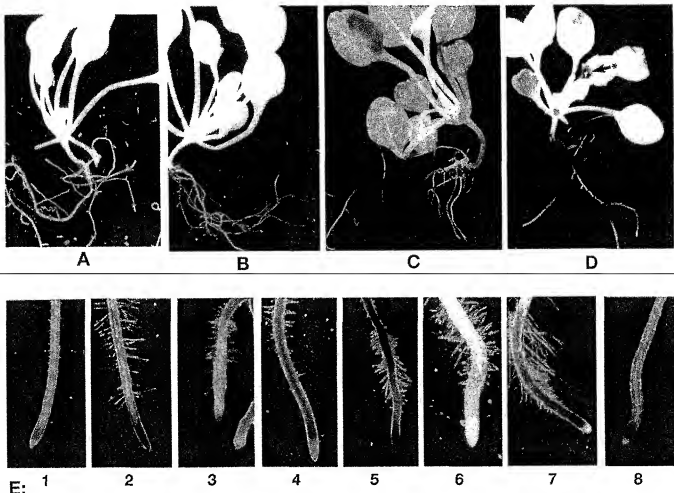


Figure 5. Tissue-specific expression patterns in transformed plants treated by environmental stresses. A, B, C, and D, GUS expression patterns in uninduced control plants and in plants treated by hypoxia, dehydration, and cold stress, respectively. The arrow in D shows that *Adh*-promoter-driven GUS expression can be found in wounded leaf areas. E, Changes in tissue-specific expression patterns in roots. 1 and 2, ADH staining pattern of uninduced and hypoxically induced roots, respectively. 3 and 4, GUS-stained roots from uninduced (3) and hypoxically induced (4) plants. 5, 6, 7, and 8, GUS staining patterns of roots from uninduced G-box-2, uninduced GT motif, and hypoxically induced GT and GC motif transformants, respectively. The length of the cell elongation zone varies a lot among different roots of one plant and depends on the growth conditions and the developmental stage of the roots. To prevent oversaturation of stainings, GUS stainings were usually stopped after 4 h. ADH staining was much faster than GUS staining, and was stopped after 10 to 15 min. Prolonged ADH staining led to the detection of background staining in control stainings without ethanol as substrate.

stress-treated plants (less frequently in plants under hypoxia; Fig. 5, A–D). The *Adh* promoter can be induced in leaves by mechanical wounding (R. Dolferus and G.L. de Bruxelles, unpublished data), another response that might be mediated by ABA (Hildmann et al., 1992).

Tissue and developmental expression patterns for the 5' deletions and site-specific promoter mutants were analyzed by *in vivo* GUS staining. It was not possible to say much about deletions from –384 onward because of the low expression levels of the constructs. The same was true for the GT and GC motif mutants. For other substitution mutants and deletions we found that, as with the CADH promoter, expression was not detectable in leaves, either in plants that had not been induced or in plants that had been challenged by any of the three stresses.

The four promoter mutants did not show any differences in GUS staining compared to CADH-GUS in the aerial plant parts, but quantitative and qualitative changes were found in the root-specific pattern (Fig. 5E). The G-box-1 mutants showed high expression following hypoxic induction and low expression following dehydration and cold treatment. There were no obvious changes in tissue specificity compared to the CADH-GUS construct. The G-box-2 mutants, as predicted by the mRNA results, showed higher uninduced expression levels and a staining pattern normally seen for induced roots (Fig. 5E [5]), with high expression in the root tip, elongation zone, and around the vascular tissues; this pattern remained unchanged after treatment with the three stresses. The GT and GC motif mutants showed expression patterns similar to each other. Under uninduced conditions, GUS activity was not detectable in the majority of transformants (Fig. 5E [6]). After hypoxic induction, GUS staining was found in the elongation zone, in lateral root primordia, and along the vascular bundles (only in the older root parts), but activity was not found in the root tip (Fig. 5E [7 and 8]). The same pattern was found in roots of dehydration- and cold-stress-treated plants, but at a lower intensity. These data indicate that the GT and GC motifs are critical for the expression of the *Arabidopsis Adh* promoter. Mutation of either region strongly reduces the uninduced expression pattern in the root, and stress-induced expression is observed mainly in the root elongation zone and around the vascular bundles.

DISCUSSION

The induction of the maize *Adh1* gene by anaerobic stress has been well documented (for review, see Freeling and Bennett, 1985), and regulatory elements of the *Adh1* promoter involved in anaerobic regulation have been well characterized (Olive et al., 1991a, 1991b). More recently it was shown that another maize anaerobic protein, Suc synthase, is also inducible by low-temperature conditions (Crespi et al., 1991). Although it is not known whether all maize anaerobically inducible genes are also induced by cold stress, the question arises whether regulation of gene expression by both environmental stresses (anaerobiosis and cold) are mediated through the same signal-transduction pathway.

To investigate more thoroughly the regulation of *Adh* gene expression by stress and to determine the number of signal-

transduction pathways that are involved in environmental regulation of gene expression, we have investigated *Adh* gene regulation in the model plant *Arabidopsis*.

As we have shown in Figure 3, the *Arabidopsis Adh* promoter is not only induced by anaerobiosis and cold stress, but also by dehydration stress. Both dehydration and cold stress have been correlated with increased levels of ABA in plant tissues (Guy, 1990; Hetherington and Quatrano, 1991; Jackson, 1991), but the correlation with anaerobic stress is less clear. The *Arabidopsis Adh* promoter contains the G-box-1 sequence (see introduction and Fig. 1), a regulatory sequence that is homologous to the ABA response element (Guillean et al., 1990). The G-box sequence has been found in a wide variety of plant genes to be associated with diverse expression properties, including light (*rbcS-1A*; Schindler et al., 1992a, 1992b, 1992c, 1992d) and UV-inducible genes (chalcone synthase; Schulze-Lefert et al., 1989; Weisshaar et al., 1991). It is also found in wheat histone genes (Tabata et al., 1991) and in ABA-inducible genes (Guillean et al., 1990). Leu zipper (b-ZIP)-type transcription factors interact with the G-box (Schindler et al., 1992b, 1992c). Gel retardation and protein purification studies have shown that the *Arabidopsis Adh* G-box-1 sequence interacts with a nuclear protein (DeLisle and Ferl, 1990; McKendree et al., 1990).

Our functional analysis of *Arabidopsis Adh* has shown that the promoter consists of a complex series of regulatory sequences or cis-acting promoter elements (Fig. 6). One of the regions, the ARE, is critical for the response of the *Adh* promoter to three environmental stresses. This region contains both the GT and GC motifs, homologous to the GT and GC motifs of the maize *Adh1* ARE sequence. Mutations in either motif give essentially the same pattern, indicating that

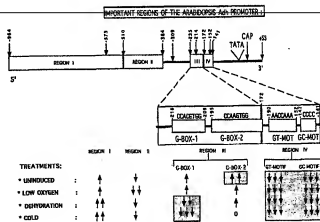


Figure 6. Summary of 5'-deletion and site-specific mutagenesis results for the *Arabidopsis Adh* promoter. Four functionally important regions were identified in the –964 to +53 CADH promoter fragment (boxes with roman numerals). Regions III and IV are shown enlarged to indicate the four regions known as *in vivo* footprinting areas (shaded boxes) and the target sequences for site-specific mutagenesis (G-box-1, G-box-2, GT and GC motifs). Results of 5' deletions and mutations on *Adh*-promoter-driven GUS mRNA expression are indicated for each region, and effects of particular importance are indicated by shaded boxes.

both the GT and GC motif might be part of the same *cis*-acting element. This region could bind a general transcription factor that can activate transcription through interactions with RNA polymerase or the TATA-box binding factor. The *Arabidopsis* ARE could have a broader function and act as a general stress responsive element.

Other upstream sequences differ in their effects on the expression of the *Adh* promoter under three environmental stress conditions: hypoxia, low temperature, and water stress. The binding of specific transcription factors to these upstream elements might determine the specificity of the stress response, and transcription under the various stresses may occur through interactions with the GT/GC motif binding protein(s). Our results suggest that the interactions between different DNA-binding factors may differ for the different stresses and that different signal-transduction pathways are involved.

Low-temperature and dehydration stress response are both mediated through the G-box-1 sequence (Fig. 6), and both stresses may be associated with increased levels of the phytohormone ABA in plants. We are currently investigating ABA induction of the *Adh* promoter and whether the cold- and dehydration-stress responses are mediated through a common signal-transduction pathway that involves ABA. The demonstration that promoter elements can interact differentially to mediate different responses provides the basis in *Arabidopsis* for a mutational analysis of common and discriminating elements in the signal-transduction pathway.

The *Arabidopsis Adh* G-box-1 sequence consists of a perfect palindromic sequence, 5'-CCACGTGG-3', which represents a binding site for G-box binding factors. The mutations we have introduced in this sequence (see Fig. 1B) completely abolish all binding-site requirements for GBF1 (Schindler et al., 1992c, 1992d) and also exclude binding of the G-box-like binding factor TGA1 (Schindler et al., 1992a). Furthermore, data presented by McKendree and Ferl (1992) suggest that the changes we have introduced in the G-box-1 sequence prevent interaction with GBF. According to Schindler et al. (1992a, 1992b, 1992c), the G-box-2 sequence is not a binding site for a GBF protein. Our data also suggest that G-box-1 and G-box-2 substitution mutants behave differently. It remains to be established which GBF(s) interact with the *Adh* promoter in *Arabidopsis* root tissues, and what their binding requirements are.

Hypoxia acts independently of the ABA-associated stresses (cold and dehydration) and the G-box-1 sequence. However, it does require the presence of the GT/GC sequence motifs. Additional upstream sequences determining hypoxic stress response may be present between positions -510 and -384, but they have not yet been located.

We are currently investigating whether the *Arabidopsis* equivalents of the maize hypoxically induced genes (aldolase, Suc synthase, and pyruvate decarboxylase) are induced by the same environmental stresses as the *Adh* gene, and whether the same promoter elements are present in these genes (G-box-1, GT and GC motifs). In addition, we will further investigate whether the responses of the *Adh* promoter to hypoxia and cold/dehydration stress can be separated using chimeric promoter constructs.

ACKNOWLEDGMENTS

We wish to thank H. Fitzpatrick and S. Stops for excellent technical assistance throughout the work, and Dr. M. Olive and Dr. D. Llewellyn for many useful discussions and for critically reading the manuscript.

Received November 24, 1993; accepted April 15, 1994.

Copyright Clearance Center: 0032-0889/94/105/1075/13.

LITERATURE CITED

- Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12: 8711-8721.
- Burke TJ, Callis J, Vierstra RD (1988) Characterization of a polyubiquitin gene from *Arabidopsis thaliana*. *Mol Genet J* 213: 435-443.
- Chang C, Meyerowitz EM (1986) Molecular cloning and DNA sequence of the *Arabidopsis thaliana* alcohol dehydrogenase gene. *Proc Natl Acad Sci USA* 83: 1408-1412.
- Christie PJ, Hahn M, Walbot V (1991) Low-temperature accumulation of alcohol dehydrogenase-1 mRNA and protein activity in maize and rice seedlings. *Plant Physiol* 95: 699-706.
- Crespi MD, Zabaleta EJ, Pontis HG, Salerno GL (1991) Sucrose synthase expression during cold acclimation in wheat. *Plant Physiol* 96: 887-891.
- DeLisle AJ, Ferl RJ (1990) Characterisation of the *Arabidopsis Adh* G-box binding factor. *Plant Cell* 2: 547-557.
- Dolferus R, Jacobs M (1991) Another ADH system... from *Arabidopsis thaliana*: an overview. *Mol Genet J* 213: 169-187.
- Dolferus R, Marbaix G, Jacobs M (1985) Alcohol dehydrogenase in *Arabidopsis*: analysis of the induction phenomenon in plants and tissue cultures. *Mol Genet J* 199: 256-264.
- Dolferus R, Van Den Bossche D, Jacobs M (1990) Sequence analysis of two null-mutant alleles of the single *Arabidopsis Adh* locus. *Mol Genet J* 224: 297-302.
- Ferl RJ, Laughner BH (1989) In vivo detection of regulatory factor binding sites of *Arabidopsis thaliana Adh*. *Plant Mol Biol* 12: 357-366.
- Ferl RJ, Nick HS (1987) In vivo detection of regulatory factor binding sites in the 5' flanking region of maize *Adh1*. *J Biol Chem* 262: 7947-7950.
- Freeling M (1973) Simultaneous induction by hypoxia or 2,4-D of multiple enzymes specified by two unlinked genes: differential *Adh1* and *Adh2* expression in maize. *Mol Genet J* 127: 215-227.
- Freeling M, Bennett DC (1985) Maize *Adh1*. *Annu Rev Genet* 19: 297-323.
- Gilmour SJ, Artus NN, Tomashow MF (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol Biol* 18: 13-21.
- Guitinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognises an abscisic acid response element. *Science* 250: 267-271.
- Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 41: 187-223.
- Hetherington AM, Quatrano RS (1991) Mechanisms of action of abscisic acid at the cellular level. *New Phytol* 119: 9-32.
- Hildmann T, Ebner H, Peña-Cortés H, Sánchez-Serrano JJ, Willmitzer L, Frei S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell* 4: 1157-1170.
- Howard EA, Walker JC, Dennis ES, Peacock WJ (1987) Regulated expression of an alcohol dehydrogenase-1 chimeric gene introduced into maize protoplasts. *Planta* 170: 535-540.
- Hwang S-Y, VanToal TT (1991) Abscisic acid induces hypoxia tolerance in corn. *Plant Physiol* 97: 593-597.
- Jackson MB (1991) Regulation of water relationships in flooded plants by ABA from leaves, roots and xylem sap. In WJ Davies, HG Jones, eds. *Environmental Plant Biology Series: Abscisic Acid, Physiology and Biochemistry*. Bios Scientific Publishers, Oxford, UK, pp 217-226.

- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5: 387-405
- Jones KA, Tian R (1985) Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro. *Nature* 317: 179-182
- Kunkel TA, Roberts JD, Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* 154: 367-382
- Ling V, Palva ET (1992) The expression of a *rab*-related gene, *rab18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. *Plant Mol Biol* 20: 951-962
- Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio/Technology* 9: 963-967
- Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. *Anal Biochem* 163: 16-20
- Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- McDonnell RE, Clark RD, Smith WA, Hinchey MA (1987) A simplified method for the detection of neomycin phosphotransferase II activity in transformed plant tissues. *Plant Mol Biol Rep* 5: 380-386
- McKendree WL, Feri RJ (1992) Functional elements of the *Arabidopsis Adh* promoter include the G-box. *Plant Mol Biol* 19: 859-862
- McKendree WL, Paul A-L, DeLisle AJ, Feri RJ (1990) In vivo and in vitro characterisation of protein interactions with the dyad G-box of the *Arabidopsis Adh* gene. *Plant Cell* 2: 207-214
- Okimoto R, Sachs M, Porter E, Freeling M (1980) Patterns of polypeptide synthesis in various maize organs under hypoxia. *Planta* 150: 89-94
- Olive MR, Peacock WJ, Dennis ES (1991a) The anaerobic responsive element contains two GC-rich sequences essential for binding a nuclear protein and hypoxic activation of the maize *Adh1* promoter. *Nucleic Acids Res* 19: 7053-7060
- Olive MR, Walker JC, Singh K, Dennis ES, Peacock WJ (1990) Functional properties of the anaerobic responsive element of the maize *Adh1* gene. *Plant Mol Biol* 15: 593-604
- Olive MR, Walker JC, Singh K, Ellis JG, Llewellyn D, Peacock WJ, Dennis ES (1991b) The anaerobic responsive element. *Plant Mol Biol* 2: 673-684
- Ooms G, Hooykaas JJ, Van Veen RJM, Van Beelen P, Regensburg-Tuinik TJG, Schilperoord RA (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. *Plasmid* 7: 15-29
- Paul A-L, Feri RJ (1991) In vivo footprinting reveals unique cis-elements and different modes of hypoxic induction in maize *Adh1* and *Adh2*. *Plant Cell* 3: 159-168
- Schindler U, Beckmann H, Cashmore AR (1992a) TGA1 and G-box binding factors: 2 distinct classes of *Arabidopsis* leucine zipper proteins compete for the G-box-like element TGACGTGG. *Plant Cell* 4: 1309-1319
- Schindler U, Menkens AE, Beckmann H, Ecker JR, Cashmore AR (1992b) Heterodimerisation between light-regulated and ubiquitously expressed *Arabidopsis* GBF b-ZIP proteins. *EMBO J* 11: 1261-1273
- Schindler U, Menkens AE, Cashmore AR (1992c) GBF-1, GBF-2 and GBF-3: three *Arabidopsis* b-ZIP proteins that interact with the light-regulated *rice5-1A* promoter. In: JL Wray, ed. *Society for Experimental Biology Seminar Series 49: Inducible Plant Promoters, Their Biochemistry and Molecular Biology*. Cambridge University Press, Cambridge, UK, pp 289-304
- Schindler U, Terzaghi W, Beckmann H, Kadesh T, Cashmore AR (1992d) DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor GBF1. *EMBO J* 11: 1275-1289
- Schulze-Lefert P, Dangel J, Becker-Andre M, Hahlbrock K, Schulz W (1989) Inducible in vivo DNA fingerprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO J* 8: 651-656
- Tabata T, Nakayama T, Mikami K, Iwabuchi M (1991) HBP-1a and HBP-1b: leucine zipper-type transcription factors of wheat. *EMBO J* 10: 1459-1467
- Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci USA* 85: 5536-5540
- Walker JC, Howard EA, Dennis ES, Peacock WJ (1987) DNA sequences required for anaerobic expression of the maize *Adh1* gene. *Proc Natl Acad Sci USA* 84: 6624-6629
- Weishaar B, Armstrong GA, Block A, da Costa e Silva O, Hahlbrock K (1991) Light inducible and constitutively expressed DNA-binding proteins recognising a plant promoter element with functional relevance in light responsiveness. *EMBO J* 10: 1777-1786

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

Paper No. 21

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte DAVID McELROY,
EMIL OROZCO, and LUCILLE LACCETTI

Appeal No. 2003-0936
Application No. 09/532,806¹

ON BRIEF



Before WINTERS, GRON, and GREEN, Administrative Patent Judges.
GRON, Administrative Patent Judge.

DECISION ON APPEAL UNDER 35 U.S.C. § 134

Introduction

This is an appeal under 35 U.S.C. § 134 of an examiner's final rejections of Claims 1, 4-54, and 85-131, all claims pending in Application 09/532,806, under 35 U.S.C. § 112, first paragraph. Claims 1, 4-54, and 85-113 stand finally rejected for noncompliance with the written description requirement of 35 U.S.C. § 112, first

¹ Application for patent filed March 21, 2000.

paragraph. Claims 1, 4-54, and 85-131 stand finally rejected for noncompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph (Brief on Appeal, p. 3 (AB 3); Examiner's Answer, p. 3 (EA 3)). Claims 1, 4-20, 33, 34, 45, 46, 48, 49, 51, 52, 85, 96, and 114, reproduced below, are representative of the full scope of the subject matter claimed.

1. An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.
4. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 110 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
5. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 125 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
6. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 250 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
7. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 400 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
8. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 750 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
9. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1000 to about

3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

10. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
11. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
12. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
13. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 3000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
14. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises the nucleic acid sequence of SEQ ID NO:1.
15. The isolated nucleic acid of claim 1, further comprising an enhancer.
16. The isolated nucleic acid of claim 15, wherein said enhancer comprises an intron.
17. The isolated nucleic acid of claim 15, wherein said intron is selected from the group consisting of the rice actin 1 intron and the rice actin 2 intron.
18. The isolated nucleic acid of claim 1, further comprising a terminator.
19. The isolated nucleic acid of claim 18, wherein said terminator comprises an rbcS terminator.

20. A transgenic plant stably transformed with a selected DNA comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.
33. The transgenic plant of claim 20, wherein said selected DNA further comprises a selected heterologous coding region operably linked to said GRP promoter.
34. The transgenic plant of claim 33, wherein said selected heterologous coding region encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, male sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.
45. The transgenic plant of claim 20, further defined as a monocotyledonous plant.
46. The transgenic plant of claim 45, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane.
48. The transgenic plant of claim 20, further defined as a dicotyledonous plant.
49. The transgenic plant of claim 48, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, cotton, canola, alfalfa, sunflower, and cotton.²

² We note here that the Markush grouping of dicotyledonous plants twice recites cotton. Counsel should edit all of appellants' claims for typographical errors.

51. The transgenic plant of claim 20, further defined as a fertile R_0 transgenic plant.
52. A seed of the fertile R_0 transgenic plant of claim 51, wherein said seed comprises said selected DNA.
85. A transgenic plant³ stably transformed with a selected DNA comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.
96. The transgenic plant cell of claim 85, wherein said selected DNA further comprises a selected coding region operably linked to said maize GRP promoter.
114. A method of preparing a transgenic plant comprising the steps of:
- (i) obtaining a construct comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1;
 - (ii) transforming a recipient plant cell with said construct; and
 - (iii) regenerating said recipient plant cell to obtain a transgenic plant transformed with said construct.

According to the examiner, (1) appellants' claims are drawn to "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention" (EA 3); and

³ Note that claims dependent upon Claim 85 refer to "[t]he transgenic plant cell of claim 85" (emphasis added).

(2) "the specification, while being enabling for the isolated nucleic acid that is the maize GRP promoter described in Example 1, transgenic plants and cells comprising said promoter, and method of preparing said transgenic plant, does not reasonably provide enablement for other isolated nucleic acids that are a maize GRP promoter, or transgenic plant, seeds and cells comprising other isolated nucleic acids" (EA 3). In support of the final rejections, the examiner cites Kim, Y., et al., "A 20 nucleotide upstream element is essential for the nopaline synthase (nos) promoter activity," Plant Molecular Biology, Vol. 24, pp. 105-117 (1994), and Benfey, P., et al., "The Cauliflower Mosaic Virus 35S Promoter: Combinatorial Regulation of Transcription in Plants," Science," Vol. 250, pp. 959-966 (1990).

Discussion

1. Rejection for inadequate written description

Figure 4 describes SEQ ID NO:1 by naming its 3536 contiguous nucleotides. In so doing, appellants' specification, as originally filed, prima facie described each and every isolated 95, 110, 125, 250, 400, 750, 1000, 1500, 2000, 2500, and 3000 contiguous nucleotide segment of the 3536 contiguous nucleotides of SEQ ID NO:1 comprising a functional maize GRP promoter. Nevertheless, the examiner is concerned that appellants' specification does not

identify which of all the possible at least 95 contiguous nucleotide segments of the 3536 contiguous nucleotides of SEQ ID NO:1 described are required to promote expression of the various coding regions to be linked thereto. More specifically, the examiner argues (EA 4-5):

35 U.S.C. § 112 requires that Appellants describe in some manner the structure of the nucleic acid sequences that would have promoter function. Showing how or why the claimed promoter sequences function, in particular showing which structural features are necessary for the function of the maize GRP promoter, is one way in which Appellants may describe the structure of subfragments of SEQ ID NO:1 that would have promoter function. Appellants might also describe the structure of subfragments of SEQ ID NO:1 that would have promoter function by describing a representative number of species of subfragments having promoter function, so that one skilled in the art would have a basis for recognizing the characteristics of SEQ ID NO:1 subfragments that retain promoter function. Here Appellants have done neither. Appellants describe only a single element that has promoter function. However, this element has only been shown to have promoter activity when linked to the rice act 2 intron 1 deletion derivative. This element appears to be a 639 base pair subfragment of the 3536 base pair sequence of SEQ ID NO:1 (examples 2 and 3, pages 112-114 of the specification, and figures 1 and 2), though it is unclear exactly which nucleotides of SEQ ID NO:1 provided this promoter function, as the specification describes the construction of the promoter containing construct only in terms of the restriction enzymes used to subclone the subfragment of SEQ ID NO:1 into the reporter construct. . . . If a nucleotide required for promoter function is not present in a given sequence, that sequence will no longer exhibit promoter function.

Unlike the specification which describes every subfragment of SEQ ID NO:1 that is between 95 and 3536 contiguous bases long, the

examiner argues that "Claim 1 encompasses every subfragment of SEQ ID NO:1 that is between 95 and 3536 contiguous bases long and that has promoter function" (EA 5; emphasis added). According to the examiner, "the description must allow those skilled in the art to recognize what regions of SEQ ID NO:1 would need to be retained in its subfragments such that the subfragments could reasonably be expected to retain promoter function" (EA 6).

As we understand the rejection, the examiner concedes that appellants' specification describes every subfragment claimed which can function as a promoter. However, that description does not satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, because the subfragments of SEQ ID NO:1 between 95 and 3536 contiguous bases in length that can function as a promoter are not distinguished from the subfragments of SEQ ID NO:1 between 95 and 3536 contiguous bases in length that cannot function as a promoter. The problem with the examiner's position is that it confuses the written description requirement of 35 U.S.C. § 112, first paragraph, with the enablement requirement of 35 U.S.C. § 112, first paragraph. For example, in support of the written description requirement of 35 U.S.C. § 112, first paragraph, the examiner states (EA 5-6) (emphasis added):

While it is not required that Appellant describe exactly the subject matter claimed, the description must allow those skilled in the art to recognize what regions of SEQ ID NO:1 would need to be retained in its subfragments such that the subfragments could reasonably be expected to retain promoter function. The Examiner maintains that the instant disclosure does not allow those skilled in the art to recognize what regions of SEQ ID NO:1 would need to be retained in its subfragments such that the subfragments could reasonably be expected to retain promoter function

The examiner's criticism that the specification would not have allowed persons skilled in the art to recognize what nucleotide subfragments of SEQ ID NO:1 function as promoters is indistinct from the concomitant criticism that the specification would not have allowed persons skilled in the art to make and use the nucleotide subfragments of SEQ ID NO:1 as promoters without undue experimentation. However, Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991), instructs at 1562, 19 USPQ2d at 1117:

This court in [In re] Wilder[, 736 F.2d 1516, 1520, 222 USPQ 369, 372 (Fed. Cir. 1994), cert. denied, 469 U.S. 1209 (1985),] (and the CCPA before it) clearly recognized, and we hereby reaffirm, that 35 USC 112, first paragraph, requires a "written description of the invention" which is separate and distinct from the enablement requirement. The purpose of the "written description" requirement is broader than to merely explain how to "make and use"; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention.

The invention is, for purposes of the "written description" inquiry, whatever is now claimed.

More recently, the Federal Circuit discussed both the Vas-Cath and Eli Lilly decisions (Vas-Cath, Inc. v. Mahurkar, *supra*; and Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997)), in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. 1316, 63 USPQ2d 1609 (Fed. Cir. 2002). The court stated in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. at 1324, 63 USPQ2d at 1613:

In Eli Lilly, we concluded that a claim to a microorganism containing human insulin cDNA was not adequately described by a statement that the invention included human insulin cDNA. *Id.* at 1557, 43 USPQ2d at 1405. The recitation of the term human insulin cDNA conveyed no distinguishing information about the identity of the claimed DNA sequence, such as its relevant structural or physical characteristics. *Id.* We stated that an adequate written description of genetic material "'requires a precise definition, such as by structure, formula, chemical name, or physical properties,' not a mere wish or plan for obtaining the claimed chemical invention," and that none of those descriptions appeared in that patent. *Id.* at 1566, 43 USPQ2d at 1404 The specification in the Eli Lilly case did not show that the inventors had possession of human insulin cDNA.

However, the court in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. at 1329, 63 USPQ2d at 1616-17, clarified:

It is true that in Vas-Cath, we stated: "The purpose of the 'written description' requirement is broader than to merely explain how to 'make and use'; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she

was in possession of the invention." Vas-Cath, 935 F.2d at 1563-64, 19 USPQ2d at 1117. That portion of the opinion in Vas-Cath, however, merely states a purpose of the written description requirement, viz., to ensure that the applicant had possession of the invention as of the desired filing date. It does not state that possession alone is always sufficient to meet that requirement. Furthermore, in Lockwood v. American Airlines, Inc., we rejected Lockwood's argument that "all that is necessary to satisfy the description requirement is to show that one is "in possession" of the invention. 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Rather, we clarified that the written description requirement is satisfied by the patentee's disclosure of "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention." Id.

Accordingly, we espouse the view expressed in Evans v. Eaton, 20 U.S. (7 Wheat.) 356 (1822), as did the court in Vas-Cath, Inc. v. Mahurkar, 935 F.2d at 1561, 19 USPQ2d at 1114-15, that:

[T]he Court concluded that the specification of a patent had two objects, the first of which was "to enable artizans to make and use [the invention]. . . ." [Evans v. Eaton,] . . . at 433. The second object of the specification was

to put the public in possession of what the party claims as his own invention, so as to ascertain if he claims anything that is in common use, or is already known, and to guard against prejudice or injury from the use of an invention which the party may otherwise innocently suppose not to be patented. It is, therefore, for the purpose of warning an innocent purchaser, or other person using . . . [the invention], of his infringement of the patent; and at the same time, of taking from the inventor the means of practicing upon the credulity or the fears of other persons, by

pretending that his invention is more than what it really is, or different from its ostensible objects, that the patentee is required to distinguish his invention in his specification.

Id. at 434.

In that light, we find that here the isolated nucleic acid maize GRP promoter appellants claim comprising at least 95, 110, 125, 250, 400, 750, 1000, 1500, 2000, 2500, 3000, and 3536 contiguous nucleotide fragments of the 3536 contiguous nucleotides of SEQ ID NO:1 of Figure 4 is so precisely defined in terms of structure, formula, chemical name, and function, including Figures 1-4 and examples, that persons skilled in the art immediately would have understood what appellants claim as their invention and could readily distinguish what appellants claim from anything that is in common use, all that is known, and anything proposed for production and use in the art. If an isolated nucleotide sequence comprises at least 95 contiguous nucleotides of the 3536 contiguous nucleotides of SEQ ID NO:1 and functions as a GRP promoter when operably linked to a coding region, appellants claim it.⁴ Whether the inventors "had possession of the claimed

⁴ The examiner has not adequately explained why appellant's Claims 14, 32, and 95 stand rejected under 35 U.S.C. § 112, first paragraph, for inadequate written description. As per those claims, the GRP promoter comprises the complete nucleic acid sequence of SEQ ID NO:1.

invention" (EA 3), the test the examiner used to determine compliance with the written description requirement of the first paragraph of Section 112, is not the gauge for compliance. As the court instructs in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. at 1330, 63 USPQ2d at 1617:

A showing of "possession" is ancillary to the statutory mandate that "[t]he specification shall contain a written description of the invention," and that requirement is not met if, despite a showing of possession, the specification does not adequately describe the claimed invention.

Accordingly, the examiner erred in rejecting Claims 1, 4-54, and 85-113 for noncompliance with the written description requirement of 35 U.S.C. § 112, first paragraph.

2. Rejection for nonenablement

The examiner's rejection of Claims 1, 4-54, and 85-131, all claims pending in this application, for noncompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, is an entirely distinct issue. At the onset, appellants' own specification teaches that: (1) the art is unpredictable, and (2) a considerable amount of experimentation may be required to enable persons skilled in the art to make and use the full scope of the subject matter claimed; yet the kind and amount of experimentation required to enable one skilled in the art to make

and use the full scope of the subject matter claimed is well within the knowledge and skill of a person with the ordinary level of knowledge and skill in this art to perform without undue experimentation. For example, appellants' specification teaches (Spec., pp. 12-13; emphasis added)):

[T]he current invention includes sequences which have been derived from the maize GRP promoter disclosed herein. One efficient means for preparing such derivatives comprises introducing mutations into the sequences of the invention, for example, the sequence given in SEQ ID NO:1. Such mutants may potentially have enhanced or altered function relative to the native sequence or alternatively, may be silent with regard to function.

Mutagenesis may be carried out at random and the mutagenized sequences screened for function in a trial-by-error procedure. Alternatively, particular sequences which provide the ZMGRP promoter with desirable expression characteristics could be identified and these or similar sequences introduced into other related or non-related sequences via mutation. Similarly, non-essential elements may be deleted without significantly altering the function of the elements. It further is contemplated that one could mutagenize these sequences in order to enhance their utility in expressing transgenes in a particular species, for example, maize.

The means for mutagenizing a DNA segment encoding a ZMGRP promoter sequence of the current invention are well-known to those of skill in the art. Mutagenesis may be performed in accordance with any of the techniques known in the art, such as, but not limited to, synthesizing an oligonucleotide having one or more mutations within the sequence of a particular regulatory region. In particular, site-specific mutagenesis is a technique useful in the preparation of promoter mutants, through specific mutagenesis of the underlying DNA. The technique further

provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. . . .

The specification teaches that "the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications" (Spec., p. 14). "The preparation of sequence variants of the selected promoter DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of DNA sequences may be obtained" (Spec., p. 15) (emphasis added). According to the specification (Spec., pp. 15-16) (emphasis added):

Examples of such methodologies are provided by U.S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety. A number of template dependent processes are available to amplify the target sequences of interest present in a sample, such methods being well known in the art and specifically disclosed herein below.

One efficient, targeted means for preparing mutagenized promoters or enhancers relies upon the identification of putative regulatory elements within the target sequence. This can be initiated by comparison with, for example, promoter sequences known to be expressed in a similar manner. Sequences which are shared among elements with similar functions or expression patterns are likely candidates for the binding of transcription factors and are thus likely elements which confer expression patterns. Confirmation of these putative regulatory elements can be achieved by deletion analysis of each putative regulatory region followed by function analysis of each deletion construct

by assay of a reporter gene which is functionally attached to each construct. As such, once a starting promoter or intron sequence is provided, any of a number of different functional deletion mutants of the starting sequence could be readily prepared.

As indicated above, deletion mutants of the ZMGRP promoter also could be randomly prepared and then assayed. With this strategy, a series of constructs are prepared, each containing a different portion of the clone (a subclone), and these constructs are then screened for activity. A suitable means for screening for activity is to attach a deleted promoter construct to a selectable or screenable marker, and to isolate only those cells expressing the marker protein. In this way, a number of different, deleted promoter constructs are identified which still retain the desired, or even enhanced, activity. The smallest segment which is required for activity is thereby identified through comparison of the selected constructs. This segment may then be used for the construction of vectors for the expression of exogenous protein.

The specification also generally describes various regulatory elements (Spec., pp. 18-21), terminators (Spec., p. 21), transit or signal peptides (Spec., pp. 21-23), marker genes (Spec., pp. 23-27), and exogenous genes for herbicide resistance, insect resistance, environment or stress resistance, disease resistance, mycotoxin reduction, grain quality, etc. (Spec., pp. 27-61), which are suitable for use in modifying plant characteristics, and include citations to prior art and summaries of the state of the art. The specification thereafter discusses assays which may be employed to determine levels of expression of new transgenic DNA

constructs (Spec., pp. 61-108) and various art recognized methods suitable for plant transformation, growth, stabilization, regeneration, seed production, and breeding (Spec., pp. 69-74), including liberal citation of the prior art and discussion of the state of the art. Following the aforementioned teachings, the specification introduces the examples presented as follows (Spec., pp. 110-111) (emphasis added):

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention.

.

The current inventors have demonstrated the utility of a novel maize promoter, designated Zea Mays Glycine Rich Protein (ZMGRP) promoter, in conjunction with an intron in transgenic maize. The ZMGRP promoter comes from a gene (Genbank Acc# GI/22312) that is induced in response to water stress and wounding. The ZMGRP mRNA has been shown to accumulate in epidermal cells upon induction (Gomez et al., 1988). The ZMGRP promoter was isolated from a maize B73 genomic library and fused to the gus reporter gene, both with and without a modified intron from the rice Act2 gene (see Example 5).

Transient expression assays in microparticle bombarded maize suspension cells and in excised maize root and leaf tissue were carried out in order to determine the activity of the ZMGRP promoter. The promoter was shown to be functionally active in conjunction with a modified actin 2 (Act2) intron 1. Furthermore, the ZMGRP promoter - Act2 intron combination yielded transient expression levels that were at least 70% the level observed from the rice actin 1 (Act1) promoter - intron combination (Zhang, W., McElroy, D., Wu, R., 1991). Finally, the ZMGRP promoter - intron - gus construct was shown to express high levels of GUS protein in the leaves, stems and meristematic regions of the roots of R₀ maize plants regenerated from transformed maize callus.

Accordingly, appellants argue that the broad teachings of the specification and claims are supported by a number of specific examples of isolated DNA comprising at least 95 contiguous bases of SEQ ID NO:1 which comprise a functional maize GRP promoter. We examine those examples below.

Example 1 teaches that the inventors serendipitously isolated the ZMGRP promoter "from a maize B73 size-selected lambda genomic DNA (gDNA) library while attempting to isolate a second maize promoter, designated A3" (Spec., p. 111). Example 1 reports (Spec., p. 112):

The analysis revealed that the restriction map and hybridization pattern of the putative clone was highly similar to, but not identical to, the expected A3 pattern. Partial sequencing of the clone revealed that the 5' sequence was highly homologous, but not identical to that of the A3 5' region. A GenBank search revealed that the 4000 base pair cloned sequence shared homology in

about the 400 most 3' based pairs with an ABA-inducible genomic clone reported by Gomez et al. (1988) (GenBank Accession Number X12564).

Example 2 characterizes the ZMGRP promoter constructs used in subsequent examples of transient expression analyses and transformations therewith. Example 2 in its entirety reads (Spec., pp. 112-113) (emphasis added):

Sequence characterization of the ZMGRP promoter-containing plasmid revealed that the ~ 4.0 kb SacI insert contained a HindIII site 97 bp from the 5' end of the insert and approximately 360 bp of ZMGRP coding sequence 3' of the ATG start codon. Restriction enzyme analysis determined that there was a unique XhoI site approximately 400bp 5' of the ATG start site. The sequence around the XhoI site was determined and used to design a 5' PCR primer. A 3' PCR primer was designed to change the sequence around the ATG start site to create an NcoI site and to introduce a SmaI site 4 bp 5' of the ATG start codon. These primers were used to PCR amplify the DNA at the 3' end of the promoter from the XhoI site to the newly created NcoI site. The PCR fragment was used in a three way ligation, employing a HindIII to XhoI fragment containing the 5' ~3.2kbp part of the ZMGRP promoter region, the XhoI to NcoI fragment containing the 3' 0.4 kbp part of the ZMGRP promoter region, and the gus-nos sequence containing vector pGN73, which had been digested with HindIII and NcoI. The resulting construct was designated pZMGRP-GN73 (Fig. 2, SEQ ID NO:2)⁵. A construct designated pZMGRP-Act2-int-GN73 was made by replacing the SmaI - NcoI region of the ZMGRP promoter with a PvuII - NcoI restriction fragment from pDPG836 containing a rice Act2 intron 1 deletion derivative (Act2-int) (Fig. 1, SEQ ID NO:3)⁶.

⁵ SEQ ID NO:2 includes 8076 nucleotides (Raw Sequence Listing (Paper No. 4)).

⁶ SEQ ID NO:3 includes 9002 nucleotides (Raw Sequence Listing (Paper No. 4)).

Example 3 (Transient Expression Analysis of ZMGRP Promoter Function) describes the transient expression assays used for analysis of (Spec., p. 113):

. . . expression of the gus reporter gene (E. coli beta-glucuronidase) fused to the ZMGRP promoter with an actin 2 intron (U.S. Serial No. 09/312,304) (ZMGRP (639) act 2 pGN73, FIG. 1) or without any intron (ZMGRP (639) pGN73, FIG. 2)

Having considered the teaching in appellants' specification, we now focus on the examiner's reasons to doubt the objective truth of the statements contained therein. In re Marzocchi, 439 F.2d 220, 223-24, 169 USPQ 367, 369-70 (CCPA 1971), instructs (footnote omitted):

As a matter of Patent Office practice . . . a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

. . . Most often, additional factors, such as the teachings in pertinent references, will be available to substantiate any doubts that the asserted scope of objective enablement is in fact commensurate with the scope of protection sought and to support any demands based thereon for proof. . . . [I]t is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or

reasoning which is inconsistent with the contested statement.

To satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, for the full scope of contiguous nucleotide sequences appellants claim,

. . . the specification [must] . . . enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without "undue experimentation." In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." Id. at 736-37, 8 USPQ2d at 1404.

In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991). As explained in In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404 (footnotes omitted), "Enablement is not precluded by the necessity for some experimentation such as routine screening." In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404, amplified the statement with a quote from Ex parte Jackson, 217 USPQ2d 804, 807 (Bd. App. 1982) (emphasis added):

The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404, instructs (footnote omitted):

Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in Ex parte Forman[, 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986)]. They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

There appears to be minimal differences in appellants' and the examiner's respective views of the evidence relative to each of the factors material to their respective determinations whether or not appellants' specification would have required persons skilled in the art to make and use the full scope of the claimed invention without undue experimentation. On balancing the weight of the collective evidence relating to all the material factors, the scales do not significantly sway one way or another. Appellants and the examiner appear to agree that, for any person skilled in the art to make and use the full scope of the claimed invention, a considerable amount of trial and error experimentation would be required. However, the specification provides a considerable amount of direction and guidance in that effort and cites prior art which suggests that the kind and amount of experimentation are routine. Appellants point to their working examples, but the examiner finds the whole and only one functionally effective 639 bp

fragment of the whole 3536 bp nucleotide appellants describe, and the 639 bp fragment is defined solely in terms of its enzymatic restriction sites. The number of promoters which have been used to effect expression of phenotypic genetic codes appears to be small, yet appellants serendipitously have discovered one which preferably promotes expression of a gene to which it is fused only when a plant transformed by the construct is stressed and disclosed its sequence and functional properties in the application before us. The claims are narrowly limited to promoters having 3536 contiguous named nucleotides and functionally effective fragments thereof having at least 95 contiguous nucleotides. The prior art cited by the examiner relating to the activity and requisite functional sequences of the cauliflower mosaic virus 35S and nopaline synthase (nos) promoters does show that the nucleotide structure essential for promoter activity is highly unpredictable. However, the same prior art shows that the level of skill and knowledge in this art is extremely high, that mutations and deletions to the basic sequence more often than not lower rather than eliminate promoter activity, and that it is well within the ordinary skill of the artisan to determine those nucleotide sequences which are critical for functional activity.

Having considered all the evidence, we are not convinced that the examiner has satisfied her burden to show that, in light of the guidance and direction provided by appellants' specification, the kind and amount of experimentation required of one skilled in the art to make and use the full scope of the subject matter claimed is more than routine.

More significantly, however, the examiner urges (EA 8-9) (emphasis added):

Appellants need to provide sufficient guidance for one skilled in the art to determine which of the claimed subfragments of SEQ ID NO:1 would be likely to have promoter function. In the absence of such guidance, it would require undue experimentation for one skill[ed] in the art to practice the claimed invention, because the ability of a particular nucleic acid sequence to function as a promoter is highly unpredictable on the basis of nucleotide sequence information alone. . . . The examiner maintains that to provide sufficient guidance for one skilled in the art to determine which sequences have promoter function, the specification must provide some indication of what specific nucleotides the sequences must retain in order to retain promoter function. Appellants need not describe why or how the invention works in order to provide such guidance.

Also see the examiner's rationale below (EA 11):

The examiner does not assert that one skilled in the art would be without sufficient guidance in obtaining the claimed contiguous subfragments because the specification does not provide sufficient structural and functional information to prepare the recited sequences. The examiner asserts that one skilled in the art would be without sufficient guidance in recognizing the claimed contiguous subfragments that

have promoter activity because the specification does not provide sufficient structural and functional information for one skilled in the art to recognize which sequence has promoter function.

In the two quotations reproduced above, we find the origins of the examiner's reversible error in this case.

Even in an unpredictable art, this being one, it is legal error for an examiner to require an applicant to disclose a common chemical structure essential for functional activity, here the nucleotide sequence of the maize GRP promoter of SEQ ID NO:1 critical for functional activity, to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph. To enable persons skilled in the art to make and use the full scope of the subject matter claimed, here all the subfragments of SEQ ID NO:1 which are in fact active as maize GRP promoters, (1) it may not be necessary to disclose, or even know, the chemical structure essential for functional activity in order to enable any person skilled in the art to make and use the full scope of the subject matter claimed, and (2) there may not be a common chemical structure essential for functional activity of the full scope of the subject matter claimed. While each case must be considered on its own facts, we are directed to reverse the examiner's rejection of appellants' claims under 35 U.S.C. § 112, first paragraph, as explained in the

examiner's answer, by In re Angstadt, 537 F.2d 498, 503-04,
190 USPQ 214, 218-219 (CCPA 1976):

If . . . the disclosure must provide "guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction, whether the claimed product will be obtained" (emphasis in original), as the dissent claims, then all "experimentation" is "undue," since the term "experimentation" implies that the success of the particular activity is uncertain. Such a proposition is contrary to the basic policy of the Patent Act, which is to encourage disclosure of inventions and thereby to promote progress in the useful arts. To require disclosures in patent applications to transcend the level of knowledge of those skilled in the art would stifle the disclosure of inventions in fields man understands imperfectly, like catalytic chemistry. The Supreme Court said it aptly in Minerals Separation, Ltd. v. Hyde, 242 U.S. 261, 270-271 (1916) . . . :

. . . the certainty which the law requires in patents is not greater than is reasonable,
having regard to their subject matter

Appellants have broadly disclosed a class of catalyst complexes whose use they deem to be part of the invention. But for this disclosure the public may have been deprived of the knowledge In this art, the performance of trial runs using different catalysts is "reasonable," even if the end result is uncertain

We have considered the examiner's explanations, appellants' responses, and all the evidence for and against the patentability of appellants' claims under 35 U.S.C. § 112, first paragraph, in light of the guidance our reviewing courts have provided.

Accordingly, we reverse the examiner's final rejections of Claims 1, 4-54, and 85-131 for noncompliance with description and enablement requirements of 35 U.S.C. § 112, first paragraph.

Conclusion

For the reasons stated herein above, it is

ORDERED that the examiner's rejections of Claims 1, 4-54, and 85-113 for noncompliance with the written description requirement of 35 U.S.C. § 112, first paragraph, is REVERSED; and

FURTHER ORDERED that the examiner's rejections of Claims 1, 4-54, and 85-131 for noncompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, is REVERSED.

REVERSED



SHERMAN D. WINTERS)
Administrative Patent Judge)



TEDDY S. GRON)
Administrative Patent Judge)



LORA M. GREEN)
Administrative Patent Judge)

) BOARD OF PATENT
) APPEALS
) AND
) INTERFERENCES

Appeal No. 2003-0936
Application No. 09/532,806

Robert E. Hanson
FULBRIGHT & JAWORSKI LLP
600 Congress Avenue
Austin, TX 78701

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte NANCY HOUMARD, LUCILLE B. LACCETTI,
ALBERT P. KAUSCH and EMIL M. OROZCO, JR.
(hereafter Houmard)

Appeal No. 2005-0409
Application No. 09/757,089¹



Before GRON, GRIMES and GREEN, Administrative Patent Judges.

GRON, Administrative Patent Judge.

DISCUSSION, REMAND and ORDER

We have before us an appeal under 35 U.S.C. § 134 of an examiner's final rejections of Claims 1, 5-12, 17-19, 22-29, 31-35, 38, 42-51, 82, 85-92, 94-98, 101, 105-123, 126 and 127 of Application 09/757,089 under 35 U.S.C. § 112, first paragraph, for noncompliance with both its written description and enablement requirements (Examiner's Answer, p. 2 (EA2)). Claims 1 and 5-13 presently pending in Application 09/757,089 are reproduced below:

¹ Application for patent filed January 9, 2001.

1. An isolated nucleic acid comprising a maize chloroplastic F16BP aldolase promoter, said promoter comprising from 95 to 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
5. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 110 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
6. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 125 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
7. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 250 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
8. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 400 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
9. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 750 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
10. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 1000 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
11. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 1500 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

12. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 1750 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

13. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises the nucleic acid sequence of SEQ ID NO:2.

Appellants' Brief On Appeal was received in Tech Center 1600/2900 on July 9, 2003. The Examiner's Answer was mailed January 8, 2004.

On March 10, 2004, Appellants filed a Reply Brief accompanied by a copy of the August 29, 2003, decision of the Board of Patent Appeals and Interferences in Appeal No. 2003-0936 under 35 U.S.C. § 134 reversing an Examiner's Final Rejection of Claims 1, 4-54, and 85-131 of Application 09/532,806, filed March 21, 2000, under 35 U.S.C. § 112, first paragraph, also for noncompliance with its written description and enablement requirements. Application 09/532,806 of Appeal No. 2003-0936 and Application 09/757,089 of this appeal are commonly assigned to Monsanto Technology LLC. LUCILLE B. LACCETTI and EMIL M. OROZCO are named coinventors of the subject matter claimed in both applications, and each application was examined in Tech Center 1600. Claims 1 and 4-14 of Application 09/532,806 of prior Appeal No. 2003-0936 are reproduced below:

1. An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.
4. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 110 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
5. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 125 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
6. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 250 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
7. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 400 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
8. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 750 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
9. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
10. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
11. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
12. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.
13. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 3000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

14. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises the nucleic acid sequence of SEQ ID NO:1.

Appellants informed the examiner in Part VII of the Reply

Brief, Summary of the Reply (RB3-4):

The same written description and enablement issues on appeal were recently decided by the Board in U.S. Patent Appl. Ser. No. 09/532,806. The specification and claims in that case were substantively the same as in this case. Both applications claimed a maize promoter sequence including fragments of at least 95 bp of the full length sequence and were rejected by the Examiner for an alleged lack of written description and enablement. The positions taken by the Examiner were essentially the same as in this case and were handled by the same Supervisory and Primary Examiners. The Board reversed the Examiner on both rejections in the '806 application, noting that the claims had literal support in the specification and that the substantial guidance provided by the specification rendered any experimentation needed to practice the full scope of the claimed invention routine. Given that the same substantive facts and legal issues are presented on appeal here, Appellants respectfully request that the same analysis be applied by the Board and the Examiner be reversed.

The Answer fails to address the shortcomings of the written description rejection noted in Appellants' Brief.

. . . .

The examiner responded to Appellants' Reply Brief by entering it, noting that the Reply Brief with the prior decision of the Board attached had been considered, and forwarding the application upon which the present appeal is based to the Board:

The Reply Brief filed 3/10/2004 has been entered and considered. The application has been forwarded to the Board . . . for decision on the appeal.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Stuart F. Baum

We understand that inquiries whether specifications satisfy the requirements of the first paragraph of 35 U.S.C. § 112 for the full scope of the claimed subject matter are claim and fact specific. Each case must stand on its own facts. Vas-Cath Inc. V. Mahurkar, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). Nevertheless, prior decisions of the Board provide invaluable instruction and guidance, especially where, as here, there are marked similarities between the presentations of the subject matter claimed, the disclosures in the supporting specifications, and the acknowledged skill and state of the art. Moreover, the cases allegedly have common assignees, common inventors and common examiners.

It is a hallmark of our legal system that similar cases are treated similarly. We do not, and no participant in this time-honored system may, casually disregard the outcome of cases with similar facts in prior decisions. Accordingly, we enter the following order.

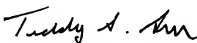


Order

It is ORDERED that Application 09/757,089 is remanded to the primary examiner in charge of prosecution for reconsideration of the prior decision of the Board in Appeal No. 2003-0936, entered August 29, 2003, and comparison of the claimed subject matter, supporting disclosures, and acknowledged skill and state of the prior art in the applications relating to Appeal 2003-0936 and this appeal; and

It is FURTHER ORDERED that after reconsidering the prior decision of the Board in Appeal No. 2003-0936, entered August 29, 2003, and comparing the claimed subject matter, supporting disclosures, and acknowledged skill and state of the prior art in the applications relating to Appeal 2003-0936 and this appeal, should the examiner maintain the rejections presently appealed, the examiner shall not only reply to each and every point raised in appellants' Reply Brief citing and/or relying on the Board's prior decision in Appeal No. 2003-0936, but the examiner also shall distinguish the facts and law of the two appeals which support a decision in this appeal contrary to our decision in Appeal No. 2003-0936.

This application, by virtue of its "special" status, requires an immediate action. Manual of Patent Examining Procedure § 708.01 (8th ed., rev. 2, May 2004). It is important that the Board be informed promptly of any action affecting the appeal in this case.

REMAND

	
TEDDY S. GRON)
Administrative Patent Judge)
)
)
ERIC BURTON GRIMES) BOARD OF PATENT
Administrative Patent Judge) APPEALS
) AND
) INTERFERENCES
LORA M. GREEN)
Administrative Patent Judge)

TSG/jlb

Appeal No. 2005-0409
Application No. 09/757,089

Robert E. Hanson
FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue,
Suite 2400
Austin, TX 78701